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Preparation of fluorescently-labeled amyloid-beta peptide assemblies: the effect of fluorophore conjugation on structure and function

L. M. Jungbauer^a, C Yu^a, K. J. Laxton^a, and M. J. LaDu^{a,*}

^aDepartment of Anatomy and Cell Biology, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA

Abstract

Recent research has focused on soluble oligomeric assemblies of the 42 amino acid isoform of the amyloid-beta peptide (A β 42) as the proximal cause of neuronal injury, synaptic loss, and the eventual dementia associated with Alzheimer's disease (AD). While neurotoxicity, neuroinflammation, and deficits in behavior and memory have all been attributed to oligomeric A β 42, the specific roles for this assembly in the cellular neuropathology of AD remain poorly understood. In particular, lack of reliable and well-characterized forms of easily detectable A β 42 oligomers has hindered study of the cellular trafficking of exogenous A β 42 by neurons *in vitro* and *in vivo*. Therefore, the objective of this study is to fluorescently label soluble oligomeric A β 42 without altering the structure or function of this assembly. Previous studies have demonstrated the advantages of using tapping mode atomic force microscopy (AFM) to characterize the structural assemblies formed by synthetic A β 42 under specific solution conditions (e.g., oligomers, protofibrils, and fibrils). Here, we extend these methods to establish a strategy for fluorescent labeling of oligomeric A β 42 assemblies that are structurally comparable to unlabeled oligomeric A β 42. To compare function, we demonstrate that the uptake of labeled and unlabeled oligomeric A β 42 by neurons *in vitro* is similar. AFM-characterized fluorophore-A β 42 oligomers are an exciting new reagent for use in a variety of studies designed to elucidate critical cellular and molecular mechanisms underlying the functions of this A β 42 assembly form in AD.

Keywords

AFM; amyloid-beta; fluorescence; assembly; oligomer; fibril; cellular uptake; Neuro-2A cells

INTRODUCTION

Intro to A β structure and function

The "amyloid hypothesis" postulates that toxicity resides in the amyloid plaques (composed of A β peptides) that develop in the extracellular space in the brains of Alzheimer's disease (AD) patients. Although extracellular amyloid plaques are a pathological hallmark of AD, soluble oligomeric assemblies of A β are most closely associated with the cognitive deficits characteristic of the disease. Thus, AD research has focused on soluble oligomeric assemblies of A β 42 as the proximal cause of *in vitro* neurotoxicity, *ex vivo* loss of neuroplasticity, and *in*

vivo deficits in behavior and memory in various experimental paradigms (Yankner and Lu, 2008). However, the specific roles of oligomeric A β 42 in the cellular neuropathology of AD remain poorly understood. In particular, cellular trafficking of exogenous oligomeric A β 42 by neurons *in vitro* and *in vivo* is critical to our understanding of mechanisms underlying its neurotoxicity. Studies of this cellular processing have been hindered by the lack of reliable and well-characterized forms of easily detectable A β 42 oligomers.

Synthetic and recombinant preparations of pure A β 42 peptide are used to understand the aggregation kinetics and behavior of the peptide, as well as to address A β structure and function. Currently, A β oligomers are defined by individual investigators using numerous methods, including neurotoxic activities, isolation technique (primarily size exclusion chromatography), size estimation by SDS or native PAGE, several imaging techniques, and reactivity with various A β conformation-specific antibodies (for review, (Rahimi *et al.*, 2008)). These studies reveal how critically dependent peptide structure is on the preparation conditions, and importantly, the close relationship between the function and structure of A β . These preparations have been used extensively to demonstrate significant functional differences between A β 42 oligomers and fibrils in a variety of experimental models (Dahlgren *et al.*, 2002; Manelli *et al.*, 2004; Trommer *et al.*, 2005; White *et al.*, 2005; Manelli *et al.*, 2007).

AFM for studying A β 42 assembly

Numerous studies have demonstrated several advantages of tapping mode AFM for A β 42 morphological characterization (Roher *et al.*, 1996; Harper *et al.*, 1997; Lambert *et al.*, 1998; Harper *et al.*, 1999; Huang *et al.*, 2000; Legleiter *et al.*, 2004; Mastrangelo *et al.*, 2006) and its folding and interactions with other proteins and membrane models (Yang *et al.*, 1999; Yip *et al.*, 2002; Chaney *et al.*, 2005; McAllister *et al.*, 2005; Choucair *et al.*, 2007; Ha *et al.*, 2007). AFM is particularly well suited to the analysis of peptides and proteins that assemble into a variety of morphologically discrete species, specifically, those like A β . AFM is one of the few techniques that provides high-resolution three-dimensional morphological images of the full range of structures formed in a single scan. In addition, *in situ* AFM studies demonstrate its capabilities for monitoring real-time aggregation kinetics of A β (Goldsbury *et al.*, 1999; Kowalewski and Holtzman, 1999; Parbhu *et al.*, 2002; Legleiter and Kowalewski, 2004; Cheng *et al.*, 2007). In our previous studies, AFM has proven very useful in developing conditions that consistently produce homogenous preparations of oligomeric or fibrillar assemblies of A β 42 (Dahlgren *et al.*, 2002; Stine *et al.*, 2003).

Fluorescent labeling of A β 42

As researchers become increasingly conscientious about utilizing structurally uniform, well-characterized A β preparations, the same criteria must be applied to derivitized A β assemblies such as fluorophore-labeled A β , prior to their widespread use as experimental tools. The sensitivity for detecting fluorophore-labeled A β facilitates a range of experimental approaches. Recent studies are using fluorophore-labeled A β 42 to elucidate mechanisms underlying the functional ability of microglia to bind and/or internalize and degrade A β 42 as a pathway contributing to A β clearance from the brain extracellular space (Paresce *et al.*, 1996; Chung *et al.*, 1999; Brazil *et al.*, 2000; Li *et al.*, 2000; El Khoury *et al.*, 2007; Majumdar *et al.*, 2007; Giunta *et al.*, 2008; Hickman *et al.*, 2008; Jiang *et al.*, 2008; Parvathy *et al.*, 2008) as well as A β 42 cellular binding, uptake and toxicity (Clifford *et al.*, 2007; Saavedra *et al.*, 2007; Simakova and Arispe, 2007; Chafekar *et al.*, 2008). Others have used fluorescent A β to examine A β -induced macrophage activation (Smits *et al.*, 2001) and transport/efflux mechanisms for blood-brain barrier A β clearance (Kuhnke *et al.*, 2007). *In vitro*, trafficking and processing of fluorescent oligomeric A β 42 by neural cells could be investigated using substantially lower concentrations of A β , thus working in the absence of acute neurotoxicity that often accompanies concentrations necessary for subsequent analysis. However, nearly all

of the fluorescent A β 42 preparations used to date originate from different preparation/solubilization methods of labeled or unlabeled A β peptide, and in many cases the peptide assemblies have not yet been structurally/morphologically characterized. Thus, structural comparisons between the unlabeled and labeled A β assemblies are not possible. Establishing the specific structural form of the assemblies, by AFM and other methods, is necessary to reliably interpret and compare results from the various fluorescent A β 42 species.

Studies using fluorescent A β 42 are facilitated by an assortment of amine-reactive fluorescent dyes and a number of N-terminally fluorophore-labeled A β 42 peptides that are commercially available (Figure 1A shows a selection of those used with A β to date). This generates the possibility for diverse and complementary approaches, but also the risk for variability, and inconsistency. For example, properties of specific fluorophores may provide unique information (such as their use as environment sensitive probes). However, as A β peptide behavior and function are highly conformation dependent, different fluorophore molecular structures such as charged groups and size may exert different effects on A β assembly and function.

The schematic in Figure 1B outlines two basic strategies for obtaining fluorophore-labeled A β 42 preparations under conditions previously characterized to generate distinct, uniform assemblies of A β oligomers and fibrils. Figure 1C summarizes considerations for each approach. In strategy A, although N-terminally labeled peptides are conveniently available from multiple commercial sources, the effect of the fluorophore on the assembly is likely variable across dyes with uncertain consequences on the final structure and/or multimer stoichiometry. In strategy B, performing the fluorophore conjugation on a stable or quasi-stable A β assembly state is straightforward, and not disruptive to the assembly process. However, an unknown degree-of-labeling requires additional experimental determination, and yield and cost may prohibit use in applications requiring high concentrations or quantity.

In this work, we examine these two strategies for preparing labeled A β assemblies, evaluating structural effects using AFM. Based on our observations, the presence of an N-terminal fluorophore generates atypical oligomer and fibril formation, whereas labeling stably formed A β assemblies leads to fluorescently labeled preparations with unperturbed structural morphology. We characterized our preparation of Alexa Fluor488[®]-labeled A β 42 oligomers using AFM and a neuronal cell uptake assay, to demonstrate that their structural morphology and functional behavior is similar to unlabeled A β 42 oligomers. Therefore, we have developed a protocol to fluorescently label and characterize assembled A β 42 oligomers as a new tool for structural and functional studies.

EXPERIMENTAL METHODS

Materials

Synthetic human amyloid-beta 1–42 was purchased from California Peptide (cat. # 641-15). For experiments comparing unlabeled and N-terminally labeled A β 42 assemblies, synthetic human amyloid-beta 1–42, HilyteFluorTM488-labeled- and FAM-labeled-amyloid-beta 1–42 peptides were purchased from Anaspec (cat. # 24224, 60479-01, 23526-01, respectively). 1,1,1,3,3,3-Hexafluoro-2-Propanol (HFIP) and anhydrous dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (cat. # 52512 and D-2650). Mica sheets and 12 mm stainless steel pucks were from Ted Pella (product # 52 and 16208, respectively). AFM probes were Al-coated Si cantilevers (42 N/m spring constant; ~300 kHz resonance frequency; tetrahedral tip with 7 nm radius (Olympus cat. # OMCL-AC160TS-W2). 1 $\times$ phosphate-buffered saline (PBS, 4 mM phosphate, 155 mM NaCl, pH (4°C) 7.5) was prepared from a 10 $\times$ stock (Invitrogen cat. # 70011). Phenol-red free Ham's F12 media was purchased from Promocell (Heidelberg, Germany, cat. C-72117) and supplemented with L-glutamine (146 g/L) prior to use. 10 mM

hydrochloric acid (HCl) was prepared from a 1 M stock (Sigma cat.# H9892). Mouse monoclonal anti-A β antibodies to residues 1–16 (6E10) and 17–24 (4G8) were from Signet/Covance.

Oligomer- and fibril-forming conditions

A β 42 oligomers and fibrils were prepared according to our previously established protocols (Stine *et al.*, 2003). Briefly, to remove pre-existing aggregates, peptide powders from commercial vendors were pre-treated by dissolving as a 1 mM solution in HFIP. Following evaporation of the HFIP in a fume hood overnight, the resulting peptide film was stored desiccated at -20°C . Immediately prior to use the films were allowed to come to room temperature (RT, $\sim 22^{\circ}\text{C}$), solubilized to 5 mM in anhydrous DMSO, sonicated in a bath sonicator (Branson, bath temperature set to 22°C) for 10 min and diluted to 100 μM in the oligomer-forming conditions (phenol-red free Hams F12, pH 7.4; incubation at 4°C for 24 h) or fibril-forming conditions (10 mM HCl, pH 2.1; incubation at 37°C for 24 h).

Preparing fluorescent oligomers and fibrils from N-terminally fluorophore labeled A β 42

Solutions of oligomers and fibrils were prepared exactly as described above for unlabeled A β 42, starting from HFIP treated peptide films prepared from the manufacturer provided lyophilized powder of FAM-A β 42 and HiLyteFluorTM488-A β 42.

Labeling A β 42 oligomers after assembly

A β 42 oligomers were prepared from HFIP-treated films as described above for unlabeled peptide, except that $1\times$ PBS was used to make the 100 μM solution instead of F12 media. After 24 h incubation at 4°C , labeling was performed using the Alexa Fluor[®]488 TFP ester Microscale Labeling Kit (A30006, Invitrogen) essentially according to manufacturer protocol. Briefly, 50 μl of 100 μM A β oligomer solution was pH adjusted to pH 9 with 5 μl of 1 M NaHCO_3 , followed by addition of 4 μl of the H_2O solubilized reactive dye. Incubation at RT ($\sim 22^{\circ}\text{C}$) was performed for 15 min followed by immediate addition of 55 μl of the labeling reaction mixture onto a spin column packed with a 425 μl slurry of Biogel P-6 resin for removal of unincorporated dye. The resulting eluant (pH 7.4) was stored for up to 2 days at 4°C or used immediately for AFM and gel/blot characterization, and cellular uptake experiments.

SDS-PAGE/Western blot characterization of labeled A β peptides

Gel electrophoresis and Western blot analysis were performed as described previously (Dahlgren *et al.*, 2002; Stine *et al.*, 2003). Briefly, samples were prepared by dilution into a non-reducing $1\times$ sample buffer (to deliver 50–100 pmoles of peptide per lane) using NuPAGE[®] 4 $\times$ LDS sample buffer (Invitrogen; 0.56 M Tris base, 0.423 M TrisHCl, 40% (w/v) glycerol, 8% (w/v) LDS, 2 mM EDTA, 0.075% (w/v) Serva Blue G250, 0.025% (w/v) Phenol Red, pH 8.5) with no heating prior to loading. SDS-PAGE was performed using NuPAGE[®] 4–12% Bis-Tris 1.0 mm gels in MES running buffer at 90–100 V, 80 mA for 80–90 min. After detection of in-gel fluorescence, Western blot transfer to 0.2 μm poly-vinylidene difluoride (PVDF) membrane was performed at 15 mA for 20–30 min followed by 25 V, 160 mA for 1 h. Transferred fluorescent peptides were again visualized on the membrane, followed by blocking in Tris-buffered saline + 0.0065% (v/v) Tween-20 (TBST) containing 5% (w/v) non-fat dry milk for 30–60 min at RT ($\sim 22^{\circ}\text{C}$). Primary antibody incubations were overnight at 4°C using mouse monoclonal antibodies 4G8 or a 4G8/6E10 mixture (at a dilution of 1:5000 each). For detection, the membrane was incubated with horseradish peroxidase-conjugated rabbit-anti-mouse IgG (1:10 000), developed using enhanced chemiluminescence (ECL) Western blotting substrate (Pierce), and visualized on the Kodak 4000R imaging system. SeeBlue[®]Plus2 Pre-Stained Standards (Invitrogen) were included for molecular weight estimation. In-gel and on-membrane fluorescence of labeled A β was visualized by excitation

with ultraviolet epi-illumination (no filters) on the Kodak 4000R system. Alternatively, fluorescence is visualized using the Typhoon 9410 variable mode imager (Amersham Biosciences) in fluorescence mode with the 532 green laser/526 SP emission filter set.

Analysis of SDS-PAGE by Silver Stain was performed according to manufacturer protocol using the Silver Xpress[®] Silver Staining Kit (Invitrogen).

Atomic force microscopy

Samples were prepared and analyzed by AFM according to previously established methods (Stine *et al.*, 2003). For samples in F12 media, freshly cleaved mica was pre-treated with 3 μ l of 1 M HCl and then rinsed with 2 drops 0.02 μ m-filtered (Whatman Anotop 10) ultrapure water, followed by immediate addition of 10 μ l of sample. For samples in HCl or PBS, 10–20 μ l of diluted sample solution was deposited directly onto freshly cleaved mica. A β solutions were diluted to 10–30 μ M in 0.02 μ m-filtered H₂O for analysis and incubated for 3 min on mica, washed with 3 drops of 0.02 μ m-filtered water and dried with tetrafluoroethane (CleanTex MicroDuster III). Samples were scanned in tapping mode under ambient conditions using a Veeco Multimode with Nanoscope IIIa controller equipped with a MultiMode head using a Vertical Engage EV piezoceramic scanner (Veeco, Santa Barbara, CA). Instrument parameters were optimized for each scan keeping contact force at a minimum, with scan rates between 1–2 Hz, drive amplitude between 20–100 mV (depending on cantilever), amplitude setpoint between 1.4 and 1.5 V. Data were processed to remove vertical offset between scan lines by zero order flattening polynomials using NanoScope Software v. 5.31 R1.

Cellular uptake

Neuro-2A (N2A) mouse neuroblastoma cells (ATCC) were seeded at 30 000 cells/well on poly-D-lysine Cellware 8-well culture slides (BD Biosciences) for 8 h in phenol-red free Dulbecco's Modified Eagle Medium (DMEM) + 10% (v/v) fetal bovine serum (FBS). Alexa488-labeled (2 μ M) or unlabeled (10 μ M) synthetic A β 42 oligomers were added to DMEM supplemented with 1% (v/v) N2 supplement (Invitrogen) and incubated for 16 h at 37°C. At the end of the treatment, cells were washed extensively with PBS and fixed in 4% (v/v) paraformaldehyde for 20 min at RT (~22°C) for imaging. Cells treated with unlabeled A β 42 oligomers were blocked for 15 min with 3% BSA (w/v), incubated overnight with anti-A β (1–16) antibody 6E10 (Covance) at 4°C, followed by 1 h incubation at RT (~22°C) with Alexa488-labeled rabbit-anti-mouse IgG (Invitrogen). Wells were mounted with VectaShield (Vector Labs) fluorescence mounting medium and covered with glass coverslips. Confocal microscopy images were acquired on a Zeiss LSM 510 META, Axiovert 200M laser scanning confocal microscope using a Plan-Apochromate Zeiss 40 $\times$ /1.3 oil immersion objective. To visualize the 488 nm excited fluorescence from the directly fluorophore-labeled A β 42 oligomers, or the Alexa488-immunolabeled A β 42 oligomers, 488 nm laser light (krypton–argon laser), a 488/543 two-notch dichroic excitation mirror, and a 505–530nm bandpass emission filter were used, with optimized photomultiplier tube (PMT) parameters. Cells cultured and treated in parallel slides to those used for confocal imaging were lysed by 15 min incubation in 35 μ l RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% SDS, Sigma-Aldrich) containing protease inhibitors (Protease Inhibitor Cocktail Set I, Calbiochem), followed by centrifugation. Supernatants were analyzed for cell-associated A β using in-gel fluorescence and western blot detection of SDS-PAGE, with loading volume normalized to total protein concentration as determined by BCA analysis. Samples were heated at 70°C for 10 min in LDS sample buffer containing 5% (v/v) β -mercaptoethanol prior to loading. Fluorescence was visualized on the Typhoon 9410. After transfer, PVDF membrane was incubated for 15 min in boiling 1 $\times$ PBS prior to blocking and antibody incubations.

RESULTS AND DISCUSSION

We examined the two approaches outlined in Figure 1B to obtain fluorescent A β 42 oligomer and fibril preparations under well-characterized conditions using three commercially available, spectrally similar fluorescent dyes. To form oligomers and fibrils starting from N-terminally labeled A β 42 (Strategy A), we used the peptide conjugated to carboxyfluorescein (FAM), and the peptide conjugated to HiLyteFluorTM488. For labeling oligomeric and fibrillar A β assemblies after their formation (Strategy B), we selected the amine-reactive TFP ester of Alexa Fluor[®]488 for its increased labeling efficacy, stability in aqueous conditions, and availability in a convenient straightforward labeling kit. Both HiLyteFluorTM488 and Alexa Fluor[®]488 are marketed as “next generation” fluorescent dyes that are brighter, more photostable and pH insensitive compared to fluorescein and its derivatives (See Figure 1A for fluorophore structures).

Strategy A: N-terminal fluorophore-labeled A β 42 forms atypical oligomers and fibrils

We purchased two different N-terminally fluorophore-labeled A β 42 peptides and performed HFIP treatment to remove pre-existing aggregate seeds according to our standard oligomer and fibril preparation protocols. We found that the specific nature of the N-terminal fluorophore affects subsequent peptide biochemistry. Under oligomer-forming conditions, HiLyte-FluorTM488-labeled A β 42 remains only partially soluble, while FAM-labeled A β 42 is soluble. Potential biochemical explanations for the affect of HiLyteFluor488 on A β 42 solubility are unknown, but may include changes to the fluorophore-peptide conjugate isoelectric point, solution conformation, and/or aggregation such that it is not stable in oligomer-forming conditions (Teplow, 2006). Brightly colored fluorescent A β 42 solutions of FAM-A β 42 oligomers and fibrils, and HiLyteFluorTM488 fibrils are readily detected as fluorescent bands by UV-excited fluorescence (Figure 2A, left) and as anti-A β antibody 4G8 signal by Western blot (Figure 2A, right) following SDS-PAGE. In addition, detection of HiLyte-A β 42 oligomers is possible by solubilization in LDS sample buffer. A typical SDS-PAGE gel analysis of our A β 42 preparations includes bands corresponding to the monomer, and a combination of bands for a dimer, trimer, and tetramer (based on molecular weight), and additional high molecular weight smears. The relative intensities of individual bands within this pattern can vary with amount loaded on the gel and method of detection. A few differences between the patterns for unlabeled and N-terminally labeled A β oligomer and fibril signals can be observed in Figure 2A. First, unlabeled A β trimer and tetramer bands are not readily detected by 4G8 compared to the FAM- and HiLyte-A β 42. In addition, the smear of high molecular weight signal ranging from approximately 40–100 kDa for unlabeled A β 42 appears from approximately 35–60 kDa for FAM- and HiLyte-A β 42. Second, although the two different N-labeled peptides have very similar molecular weight (4873.4 and 4871.5 Da for FAM and Hilyte, respectively, versus 4515.1 Da for unlabeled A β), their migration on the SDS-PAGE gel is different. N-terminal FAM-A β 42 runs just above unlabeled A β 42 while HiLyte-labeled A β 42 appears at the same position of unlabeled A β 42 (Figure 2A). These differences under denaturing SDS-PAGE analysis likely arise from N-terminal fluorophore effects A β peptide biochemical properties. Comparison of the left and right panels in Figure 2A reveals that the high molecular weight species appear absent from the fluorescence signal, likely due to fluorescence quenching in highly self-aggregated species. Finally, as demonstrated previously (Dahlgren *et al.*, 2002; Stine *et al.*, 2003), conformation-specific differences between oligomeric and fibrillar preparations of both unlabeled and N-terminally labeled A β 42 are not readily apparent in this assay (Bitan *et al.*, 2005). Thus, in general, solutions of oligomers and fibrils formed from FAM- and HiLyte-A β 42 exhibit similar behavior to the unlabeled peptide by SDS-PAGE.

To determine if the presence of the N-terminal fluorophore during oligomer and fibril formation affected the morphology of the assemblies, we characterized N-labeled fluorescent A β

preparations using AFM to compare unlabeled and N-labeled A β 42 structural assemblies. Figure 2B shows that the presence of N-terminal FAM on A β under oligomer-forming conditions causes a range of variable effects on morphology (Figure 2B, top row). Unlabeled A β 42 oligomers exhibit the typical appearance of small oligomeric structures in the absence of protofibrils and fibrils. The AFM image of FAM-A β 42 oligomers includes all types of representative morphologies observed across samples, ranging from round oligomer-like shapes similar to the unlabeled peptide (red arrow), to those exhibiting taller z height (cyan arrow), larger amorphous aggregates (green arrow), and protofibril-like aggregates (grey arrow). Relative abundance of these structures varies among samples. Because HiLyteFluorTM488-A β 42 was only partially soluble under oligomer-forming conditions, AFM analysis was not performed. The bottom row of Figure 2B shows AFM analysis of A β 42 fibrils. All three types of fibrillar assemblies appear similar in diameter and height with lengths extending over several microns. The unlabeled A β 42 fibrils exhibit the characteristic curved morphology. However, N-labeled fibrils with both the FAM and HiLyte fluorophores exhibit a straight morphology with amore rigid appearance. It also appears that individual strands tend to align in parallel. In addition, both FAM- and HiLyte-labeled fibril preparations contain short protofibril-like species (red arrow), and increased non-fibrillar small globular A β (green arrow) compared to unlabeled fibrils. Interestingly, the morphology of N-terminally fluorophore-labeled fibrils closely resembles that of fibrils formed by a mutant A β 42 peptide containing the Arctic mutation E22G (Dahlgren *et al.*, 2002). The E22G mutant fibrils also exhibited significantly greater decreases in neuronal viability compared to WT A β fibrils. Therefore, changes in fibril and oligomer morphology observed here for N-terminally fluorophore-labeled A β assemblies may alter functional properties. These data support that fluorescent A β assemblies formed from FAM-A β 42 and HiLyte-A β 42 under oligomer- and fibril-forming conditions have structural features distinct from unlabeled oligomers and fibrils.

It may be possible to avoid variable and inconsistent effects of N-labeled peptides on A β assembly, such as those observed here for FAM-labeled oligomers, by taking alternative approaches to preparing the A β . For example, mixing unlabeled and N-terminally labeled peptide prior to assembly (Chafekar *et al.*, 2008; Nielsen *et al.*, 2008), adding a flexible linker between the fluorophore and the A β N-terminus (Frost *et al.*, 2003), or using alternative initial solubilization of the peptide, such as alkaline conditions (Teplow, 2006). However, extensive structural comparisons are needed to assess whether any alternative approach is a suitable option for preparing defined fluorescent A β assemblies from N-terminally labeled starting material.

Strategy B: A β 42 oligomers labeled after formation appear similar to unlabeled oligomers

We also prepared fluorescent A β 42 assemblies by performing fluorophore conjugation after incubating the peptide under our defined assembly conditions. A β 42 oligomers were prepared using our standard protocol starting from HFIP-treated unlabeled A β 42 peptide film, with one modification: the buffer for oligomer formation was switched to 1 $\times$ PBS, pH 7.4 to avoid the presence of free amines in the buffer during labeling that would compete with the peptide for the Alexa Fluor[®]488 TFP ester. Post-labeling reaction purification using a microspin column packed with 6 kDa size-exclusion resin removes most of the unincorporated fluorophore dye, yielding a yellow-green solution of brightly fluorescent A β 42 oligomers. This process also results in sample dilution and loss of material to the beads, with estimated yields of approximately 40%. The purified Alexa Fluor[®]488-oligomer solution contains a mixture of both unlabeled and labeled A β 42 peptides, including both singly and doubly-labeled A β 42 (based on preliminary matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis, data not shown).

As shown in Figure 3A, solutions of Alexa Fluor[®]488-labeled A β 42 oligomers are readily detected using in-gel fluorescence (left), 4G8-western blot (middle), and silver stain (right). In the left panel, the fluorescent band at the position of A β 42 confirms successful covalent conjugation of Alexa Fluor[®]488 to A β 42 in the oligomer-containing solution. The three-band pattern typically observed for unlabeled A β 42 (monomer, trimer, tetramer) is also observed for Alexa488-A β 42 detected by in-gel fluorescence and Silver stain, resembling the banding pattern of unlabeled A β 42, with the 4G8-detected band at the trimer position very faint for Alexa488-A β 42. Silver stain also reveals a very faint band between the monomer and trimer bands for both types of A β that is positioned higher for unlabeled A β being higher than for Alexa Fluor[®]488-labeled A β . Similar to the N-terminally labeled A β 42 peptides, the conjugation to Alexa Fluor[®]488 appears to affect certain A β 42 peptide biochemical properties, reflected in minor differences seen by SDS-PAGE analysis. In addition, solutions of unmodified A β 42 oligomers tolerate acidic conditions, while Alexa Fluor[®]488-labeled A β 42 oligomers exhibit insolubility when the solution pH is lowered to 4 or below. Independent silver stain analysis was also performed to assess the ability of the antibodies (6E10/4G8) to detect the total A β present in the preparation (unlabeled, N-labeled and internally-labeled). A partially resolved band atop the abundant signal at the monomer position is observable depending on the detection method (see arrow in the fluorescent and silver stained gels, Figure 3A). This is potentially a different species with altered migration due to the presence of multiple Alexa488 labels on the same peptide, or anomalous migration of the same MW product (similar to observations for FAM vs. HiLyte) due to a single Alexa488 present at a different position than the major product (e.g., at either one of the two internal lysine positions (K16 and K28) instead of the N-terminus).

To determine if adding a fluorophore to assembled A β 42 oligomers affects their morphology, we characterized Alexa Fluor[®]488-labeled A β preparations using AFM. Comparison of their structural morphology to that of unlabeled A β oligomer preparations (Figure 3B) shows that both solutions contain small globular oligomers, free from large amorphous aggregates, protofibrils, or fibrils. These data support that the A β 42 oligomeric assembly state is stable to the labeling procedure and the morphology is unaffected by the fluorophore conjugation. Infrequent observation of short straight fibril-like structures appeared in some samples; however these are minor component. Similar to unlabeled oligomers, Alexa Fluor[®]488-labeled oligomer solutions can be stored for several days at 4°C or incubated for 24 h at 37°C with negligible changes in the morphology observed by AFM, and extended incubation at 37°C (48 h) leads to eventual appearance of fibril-like structures.

Alexa Fluor[®] 488-labeled A β 42 oligomers exhibit similar cellular uptake to unlabeled oligomers

We further characterized Alexa488-labeled oligomers by comparing their function to unlabeled oligomers in a cellular uptake assay. Mechanisms of A β 42 oligomer internalization by neuronal cells are poorly understood; however receptor-mediated endocytosis by a variety of receptors has been suggested (see (Verdier *et al.*, 2004) for review). As fluorophore labeling may interfere with A β 42 receptor binding and A β 42 internalization/uptake could be sensitive to its conformation, evaluation of uptake behavior for fluorophore-labeled A β assemblies is an important aspect of their characterization. We compared A β uptake using CLSM analysis of immunofluorescence for unlabeled oligomers and direct fluorescence for Alexa Fluor[®] 488 oligomers (Figure 4, Panel A). The detected fluorescence signals in cells treated with 2 μ M Alexa Fluor[®]488-labeled oligomers (left) and with 10 μ M unlabeled A β 42 (right) both appear as regions of punctate fluorescence, with large extracellular aggregates observed as bright fluorescent spots. In both cases, the majority of cells internalize A β , with fluorescent puncta appearing throughout the cell bodies. In addition, antibody-based detection of internalized A β appears to label A β localized near the perimeter of some cells (right panel); however, this

likely arises from the higher concentration of A β required for detection by antibody versus directly fluorescently labeled A β signal. We also compared cellular uptake by analyzing unlabeled and Alexa Fluor[®]488-labeled A β levels in cell lysates from cells treated in parallel (Figure 4B). Lysates from cells treated with Alexa-labeled A β oligomers (top panel) and unlabeled A β oligomers (middle panel) both show A β signal, confirming cellular uptake of both types of A β oligomers. These data suggest that oligomer properties, as measured by cellular uptake, are unperturbed by the post-assembly Alexa Fluor[®]488 fluorophore modification. Here the advantages of a fluorophore label for direct detection and sensitivity are apparent. Intracellular AlexaFluor[®]488-labeled A β 42 arising from cells treated with 2 μ M is readily detected by in-gel fluorescence, while the antibody detection fails to pick up this signal. Alexa Fluor[®]488-labeled oligomer preparations are a mixture of labeled and unlabeled peptide, indicating that the individual A β 42 subunits comprising each oligomer are not uniformly labeled. This lower degree of labeling may actually be advantageous, as the fluorophore is present at a high enough level for sensitive detection of the oligomers, but, as shown here, not to the extent of interfering with conformation and function.

A different approach is needed for preparing fluorophore-labeled fibrils

Although strategy B is a suitable approach for labeling oligomers, it cannot be applied to fibrils prepared by our methods. Fibril-forming conditions (pH 1–2) are sub-optimal for amine-based conjugation chemistry. Several modifications to the labeling protocol failed to generate labeled A β 42 fibrils comparable to unlabeled fibrils. For example, with extended reaction time the poor solubility of the Alexa Fluor[®]488-TFP ester, and of the resulting fluorophore conjugate at low pH caused the dye and fibril mixture to gel out of solution. Raising the fibril-containing solution pH to a more favorable value for amine conjugation chemistry also proved unsuccessful as the fibril morphology is highly sensitive to pH (Stine *et al.*, 2003). One possible solution for future studies is to form fibrils by extended incubation in PBS (e.g., 1 week) at 37°C and then perform the amine-reactive fluorophore-labeling reaction, similar to Parvathy *et al.* (2008). However, structural comparison of these fluorescent- fibrils to their well-characterized unlabeled fibrils has not yet been performed.

Comparison to other characterized fluorescent A β preparations

Limited characterization of other recently employed fluorophore-labeled A β preparations has been performed using various combinations of structural and functional analyses (Webster *et al.*, 2001; Saavedra *et al.*, 2007; Chafekar *et al.*, 2008). In general, within a given experimental analysis technique and A β preparation method, at least one feature of unlabeled A β is preserved in fluorophore-labeled A β preparations. For example, N-terminally fluorescein-labeled oligomers and fibrils prepared using oligomer- and fibril-forming conditions similar to our protocol showed similar morphology to the corresponding unlabeled assemblies by negative-stain transmission electron microscopy (EM) analysis, and their functional characterization revealed similar induction of neuronal apoptosis for labeled and unlabeled preparations (Saavedra *et al.*, 2007). Labeled oligomer and fibril preparations starting from a mixture of 2:1 unlabeled/tetramethylrhodamine labeled-A β 42 prepared using oligomer- and fibril-forming conditions similar to our protocol also showed comparable morphology to unlabeled assemblies by EM (Chafekar *et al.*, 2008). Functional data showed comparable behavior between unlabeled and labeled A β fibrils, but less neurotoxicity for fluorescent-oligomers compared to unlabeled oligomers (Chafekar *et al.*, 2008). In these studies, smaller less obvious changes to structure/morphology may not be readily apparent after fixing for EM, as the oligomers in these images appear clumped together, and height information is not available in these data. Furthermore, when mixtures of labeled and unlabeled peptide are used, it is unknown whether the fluorophore-labeled peptide incorporates into unlabeled assemblies. Taken together, the above results and our data emphasize the importance of characterizing A β preparations using multiple complimentary structural and functional techniques,

particularly including AFM analyses that enable comparison of detailed morphological features at high resolution.

CONCLUSIONS

AFM is an extremely useful tool to monitor possible modifications to A β structure/morphology caused by fluorophore-labeling. Our comparison of two strategies for preparing fluorescent A β 42 assemblies showed that for A β oligomers, forming the defined A β assemblies prior to fluorophore-labeling offers an approach that preserves the morphology of unlabeled A β . On the other hand, the presence of an N-terminal fluorophore during assembly exerts variable effects on A β morphology, resulting in formation of atypical oligomers and fibrils. Future studies could further examine how N-terminal fluorophore effects on A β peptide structure and biochemical properties relate to the morphological variations observed here, and may reveal additional important details on the role of the N-terminus in structure and function. Alexa Fluor®488-labeled A β 42 oligomers are directly fluorophore-labeled A β assemblies with comparable structure and function to the unlabeled assemblies and will be useful tool to elucidate important structural and functional properties of A β 42. Future studies are underway to fully characterize the fluorophore/peptide stoichiometry, and the specific location(s) of the fluorophore within individual monomer sequence as well as within the subunits comprising the oligomer assemblies. These studies will also provide useful information on the structural properties of A β oligomers. In general, applying AFM structural characterization in parallel to *in vitro* functional studies allows for observed functions of A β to be interpreted in the context of specific assembly states. This is extremely important since a primary question in AD research focuses on identifying the pathogenic A β assembly form(s).

Abbreviations

Ab	Amyloid-beta peptide
AD	Alzheimer's disease
AFM	atomic force microscopy
BCA	bicinchoninic acid
CLSM	confocal laser scanning microscopy
LDS	lithium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
PMT	photomultiplier tube
PVDF	polyvinylidene difluoride
RIPA	Radio-immunoprecipitation assay
RT	room temperature
SDS	sodium dodecyl sulfate

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(A)

Fluorophore	Structure	Commercially available as	
		N-terminal A β 42 peptide	Amine-reactive conjugate
Fluorescein (Carboxy-fluorescein = FAM; fluorescein isothiocyanate = FITC)		✓ ^a	✓ ^{a, b}
HiLyteFluor™488 (available in multiple colors)		✓ ^a	✓ ^a
AlexaFluor®488 (available in multiple colors)			✓ ^b
Tetramethylrhodamine (TAMRA)		✓ ^a	✓ ^{a, b}
Cy3/Cy5 (and derivatives)			✓ ^c

(B) Strategy A

Strategy B

(C)

STRATEGY A	STRATEGY B
Starting from HFIP treated N-terminal fluorophore-labeled A β 42 peptide under known oligomer- and fibril-forming conditions.	Labeling oligomers and fibrils after formation via amine-reactive conjugation.
PROS	
Label is already on peptide- no need for purification of free dye from oligomers.	Reduced chance of fluorophore conjugation affecting oligomer structure.
Can store labeled peptide and prepare oligomers as needed.	Numerous fluorophores available as activated dye reagents.
CONS	
May affect assembly process and resulting oligomer/fibril structure.	Labeled oligomers must be made fresh before each use.
Limited to those fluorophores stable to peptide synthesis conditions.	Reduced yield due to post-labeling purification to remove unincorporated dye.
Quenching of fluorescence a possibility upon self-association	Unknown stoichiometry of labeled:unlabeled peptide/oligomers.

R = amide bond to A β N-terminal amine or R = reactive functional group for conjugation; for HiLyteTMFluor488 R₁-R₆ = substituents. a = available from Anaspec, Inc.; b = available from Invitrogen; c = available from GE Healthcare.

Figure 1.

Fluorescent labeling of A β 42. (A) Structures of fluorophores commercially available as N-terminal A β 42 peptide conjugates and as activated reagents for conjugation that have been used in studies on A β 42. (B) Schematic of two approaches to fluorescently-labeled A β 42 assemblies. (C) Comparison of two approaches for preparing fluorescent A β 42 assemblies. This figure is available in color online at <http://www.interscience.wiley.com/journal/jmr>

Figure 2. Characterization of A β assemblies prepared from N-terminal fluorophore-labeled A β 42 peptides by SDS-PAGE/Western blot and AFM analysis. (A) Fluorescently labeled A β 42 preparations appear similar to unlabeled A β 42 by gel/blot analysis. Unlabeled (U) and N-terminally labeled A β 42 peptides (FAM-A β 42 (F) and HiLyteFluor™488-A β 42 (H)) were prepared under oligomer- and fibril-forming conditions. SDS-PAGE was performed on solutions of labeled and unlabeled oligomers and fibrils. Following transfer to PVDF membrane, analysis was performed by UV-excited fluorescence (left), and Western blot detection using anti-A β monoclonal antibody 4G8 (right). (B) AFM analysis shows distinct structural features present in oligomers and fibrils formed in the presence of N-terminal

fluorophores. Synthetic A β 42 prepared from unlabeled (U, left), N-terminal FAM-labeled (F, middle), and N-terminal HiLyteFluorTM488-labeled (H, right) were allowed to assemble under standard oligomer-forming (top row) and fibril-forming (bottom row) conditions. Solutions were then analyzed on mica under ambient conditions using tapping mode AFM. The HiLyteFluorTM488-labeled peptide was only partially soluble under oligomer-forming conditions and was therefore not analyzed by AFM. Oligomers were diluted to 10 μ M for analysis and fibrils were diluted to 30 μ M (unlabeled, HiLyteFluorTM488-labeled) and 20 μ M (FAM-labeled). All AFM images shown are 2 $\times$ 2- μ m x-y, 10 nm total z-range.

AFM images shown are $2 \times 2\text{-}\mu\text{m}$ x-y, 10 nm total z-range. This figure is available in color online at <http://www.interscience.wiley.com/journal/jmr>

Figure 4.

Following uptake, Alexa Fluor®488-labeled A β 42 oligomers appear as punctate fluorescence within the cell, similar to unlabeled A β 42 oligomers. A β uptake in Neuro-2A cells was analyzed using confocal laser scanning microscopy (A) and SDS-PAGE/Western blot analysis of cell lysates (B). Panel A shows N2A cells treated for 16 h with 2 μ M Alexa Fluor®488-labeled A β 42 oligomers (left, a) and a parallel experiment performed with 10 μ M unlabeled A β 42 oligomers, immunodetected with anti-A β monoclonal antibody 6E10 and Alexa Fluor®488-rabbit-anti-mouse (right, u). Scale bar = 44 μ m. The insets show a single-cell magnification. Scale bar = 12 μ m. Panel B shows SDS-PAGE of N2A lysates analyzed by in-gel fluorescence (top), followed by immunoblotting for A β 42 (middle), and tubulin (bottom, loading control).

Lane 1 (a) = N2A cell lysate from 2 μ M Alexa Fluor[®]488-A β oligomer treated cells; Lane 2 (u) = lysate from N2A cells treated with 10 μ M unlabeled A β oligomers; Lane 3 (a*) = ~10 pmole Alexa Fluor[®]488-labeled A β oligomer standard. Lysates in Panel B were prepared from cells treated in parallel to those analyzed by confocal microscopy in panel A.