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Interactions of Aβ(1–40) with Glycerophosphocholine and Intact Erythrocyte Membranes: Fluorescence and Circular Dichroism Studies

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Deposition of amyloid β peptide in human brain in the form of senile plaques is a neuropathological hallmark of Alzheimer's disease (AD). Levels of a phospholipid breakdown product, glycerophosphocholine (GPC), also increase in AD brain. The effect of GPC on amyloid β(1–40) peptide (Aβ) aggregation in PBS buffer was investigated by circular dichroism and fluoresence spectroscopy; interactions of Aβ and GPC with the intact erythrocyte membrane was examined by fluoresence spectroscopy. Fluorescamine labeled Aβ studies indicate GPC enhances Aβ aggregation. CD spectroscopy reveals that Aβ in the presence of GPC adopts 14% more β-sheet structure than does Aβ alone. Fluorescamine anisotropy measurements show that GPC and Aβ interact in the phospholipid head-group region of the erythrocyte membrane. In summary, both soluble Aβ and GPC insert into the phospholipid head-group region of the membrane where they interact leading to β-sheet formation in soluble Aβ which enhances Aβ aggregation.

KEY WORDS: Aβ peptide; aging; Alzheimer's disease; circular dichroism; fluorescence; glycerophosphocholine.

INTRODUCTION

Alzheimer's disease (AD) is the most common type of senile dementia (1). The neuropathological hallmark of AD is progressive deposition of amyloid β-sheet peptides in the form of senile plaques (SP) (2). Amyloid precursor protein (APP), a ubiquitous membrane protein, is cleaved to give amyloid β peptide, a 39–42 amino acid residue, 4 kDa peptide. The 40 amino acid residue peptide (Aβ) is examined in this study. Aβ and its fragments, at micromolar concentrations, are toxic to cultured neuronal cells, demonstrating a link between Aβ and AD neurochemical lesions (3). Several studies demonstrate direct interactions of Aβ with neuronal membranes (2,4,5).

Postmortem nuclear magnetic resonance (NMR) studies demonstrate that levels of degradation products of membrane phospholipids, phosphodiesters (PDE), such as glycerophosphocholine (GPC), increase in AD brain compared with control subjects (6–11); other studies demonstrate distinct changes in the mole fractions of membrane phospholipids in AD brain (12). *In vivo* ^{31}P MRS studies of AD subjects also demonstrate increased levels of PDEs in AD subjects (13). The NMR PDE findings have been confirmed by classical neurochemical analyses of AD brain which also demonstrate elevated levels of GPC

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(14). Previous light scattering studies demonstrated that GPC enhances Aβ aggregation (15). The present study examines by fluorescence spectroscopy the interaction of Aβ and GPC with the intact erythrocyte membrane to determine a possible pathophysiological role for elevated levels of GPC in AD. CD spectroscopy studies monitored the conversion of soluble Aβ from α-helical to β-sheet structures in the absence or presence of GPC. The present study also uses fluorescamine labeled Aβ to monitor Aβ aggregation in the presence or absence of GPC.

EXPERIMENTAL PROCEDURES

Materials. Aβ(1–40) was purchased from Recombinant Peptide Technologies (Atlanta, GA, USA). The naturally occurring L-isomer of GPC was purchased from Sigma (St. Louis, MO, USA). The fluoroprobes, fluorescamine, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (DPPE-ANS), 12-(9-anthroyloxy) stearic acid [12(9)AS], and 2-(3-(diphenylhexatrienyl)propanoyl) -1-hexadecanoyl-sn-glycero-3-phosphocholine (PPC-DPH) were purchased from Molecular Probes (Eugene, OR, USA).

Fluorescence Studies with Intact Erythrocyte Membranes. All fluorescence spectroscopy studies were performed on a Perkin Elmer LS55 spectrofluorometer at 25°C. Four fluorophores were used to monitor erythrocyte membrane molecular motion at different and distinct membrane sites (Fig. 1) (16) under four experimental conditions: PBS buffer only; Aβ in PBS buffer; GPC in PBS buffer; and Aβ + GPC in PBS buffer. Stock solutions of 1mM DPPE-ANS in methanol, 200 mM fluorescamine in 100% acetone, 1.0 mM 12(9)AS in 100% ethanol, and 1.0 mM PPC-DPH in 1,4-dioxane were used for fluorescence studies. The excitation (Ex) and emission (Em) frequencies used for the fluorophores are: DPPE-ANS (Ex = 340 nm, Em = 520 nm); fluorescamine (Ex = 385 nm, Em = 490 nm); 12(9)AS (Ex = 365 nm, Em = 460 nm); and PPC-DPH (Ex = 362 nm, Em = 455 nm). Aβ was dissolved in PBS, pH 7.40 to obtain a 10 μM solution, kept at 10°C overnight, and incubated 24 h at 37°C for aggregation. GPC was dissolved in PBS (pH 7.4) to obtain a 50 mM stock solution.

Each of the anisotropy experiments were carried out with the addition of the following volumes of fluorophore stock solutions and incubation times: 12 μl DPPE-ANS (30 min); 10 μl 12(9)-AS (15 min); 12 μl PPC-DPH (30 min); and 20 μl fluorescamine (9 min). We have not used whole blood for our fluorescence studies. Human blood (5 μl) was taken and diluted to 4 ml in all four cases (whole blood is diluted by 800%). Case (1) Whole blood (5 μl) was diluted to 3.9 ml of PBS buffer for the PBS buffer only experiment. Case (2) Whole blood (5 μl) was diluted with 2 ml of PBS buffer and 1.9 ml of Aβ in PBS buffer for the Aβ experiment. Case (3) Whole blood (5 μl) was diluted with 3.9 ml of PBS buffer and 80 μl of GPC stock solution for the GPC only experiment. Case (4) Whole blood (5 μl) was diluted with 2 ml of PBS buffer, 1.9 ml of Aβ stock solution and 80 μl of GPC stock solution for the Aβ + GPC experiment. The overall concentration of Aβ was 5 μM and GPC was 1 mM in respective experiments with intact erythrocytes.

Fluorescamine Aβ aggregation studies. Fluorescamine labeled Aβ was used to monitor the deposition of Aβ (Fig. 2). Fluorescamine does not fluoresce until it reacts with primary and secondary amines in soluble Aβ. Freshly prepared Aβ in PBS buffer (pH 7.4) was mixed with fluorescamine resulting in

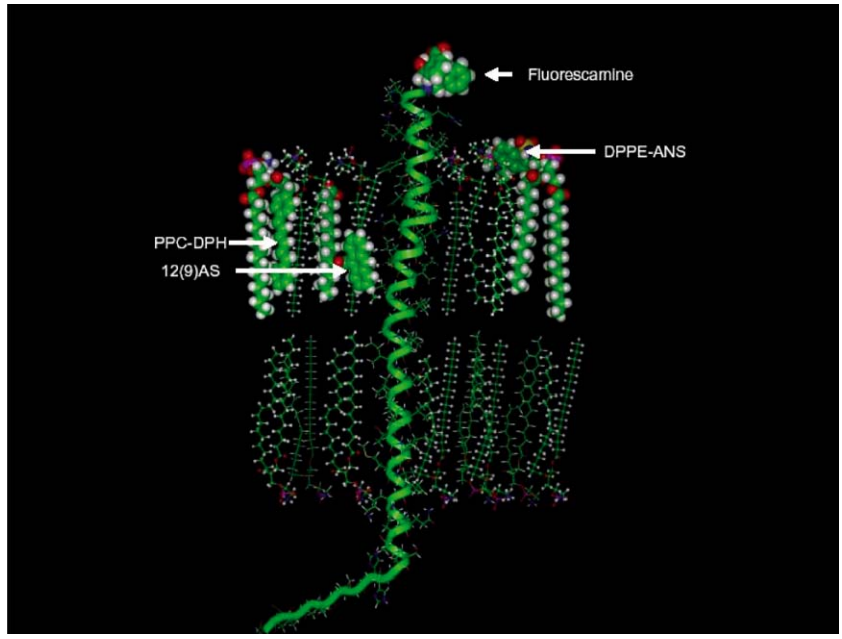

Fig. 1. Schematic showing membrane regions labeled by the fluorophores used in this study.

a 1:62.5 (Aβ: fluorescamine) mole ratio. The excitation (385 nm) and emission spectra (490 nm) were recorded with and without GPC (Fig. 2) at three time points. The overall Aβ concentration was 23 μM and the overall GPC concentration was 2 mM.

Circular Dichroism Measurements. CD spectra were recorded with an Aviv CD spectrometer at 25°C, spectral range of 190–260 nm, resolution of 0.2 nm, bandwidth of 1 nm, and a scan speed of 100 nm/min with 2 s response time. A 1 mm quartz cell was used. The overall Aβ concentration was 41 μM and GPC concentration was 540 μM in PBS buffer, pH 7.4. CD data were collected on freshly prepared solutions and after 10 days. CD data were analyzed using the CONTINLL program from a web-based software package (http://www.cryst.bbk.ac.uk/cdweb/html/home.html) (17–19).

RESULTS

Both Aβ and GPC alter the anisotropy of DPPE-ANS in the erythrocyte membrane. In addition there was a marked synergistic effect of Aβ + GPC on DPPE-ANS anisotropy (Fig. 2). The anisotropies of fluorescamine, 12(9)AS and PPC-DPH were not altered by either or Aβ + GPC and a comparative analysis of the four fluorophores is given in Fig. 3.

Figure 4 shows the fluorescence intensity (both absorption and emission) of fluorescamine-labeled Aβ in PBS buffer (pH 7.4) with and without GPC taken at 30 min, 28 h, and 118 h. The fluorescence intensity is on an arbitrary scale. The fluorescence intensity is reduced over time due to Aβ aggregation and precipitation to the bottom of the cuvette and out of the light path; the decrease in fluorescence intensity (dotted line, Fig. 4) is more pronounced in the presence of GPC than in the absence of GPC.

Figure 5 shows the CD spectra of Aβ in PBS buffer in the presence or absence of GPC taken on freshly prepared solutions (1–2 h) and at 10 days. Aβ in PBS buffer at pH 7.4 adopts a random coil conformation; however, with time, the spectra change with less negative ellipticities at wavelengths below 195 nm and more negative ellipticities at wavelengths around 216 nm, indicative of more β-sheet structure; β-sheet structure production is enhanced in the presence of GPC as evidenced by more negative ellipticity at 216 nm. Analysis of CD data (19) indicates that Aβ assumes 14% more β-sheet conformation in the presence of GPC compared to Aβ in the absence of GPC.

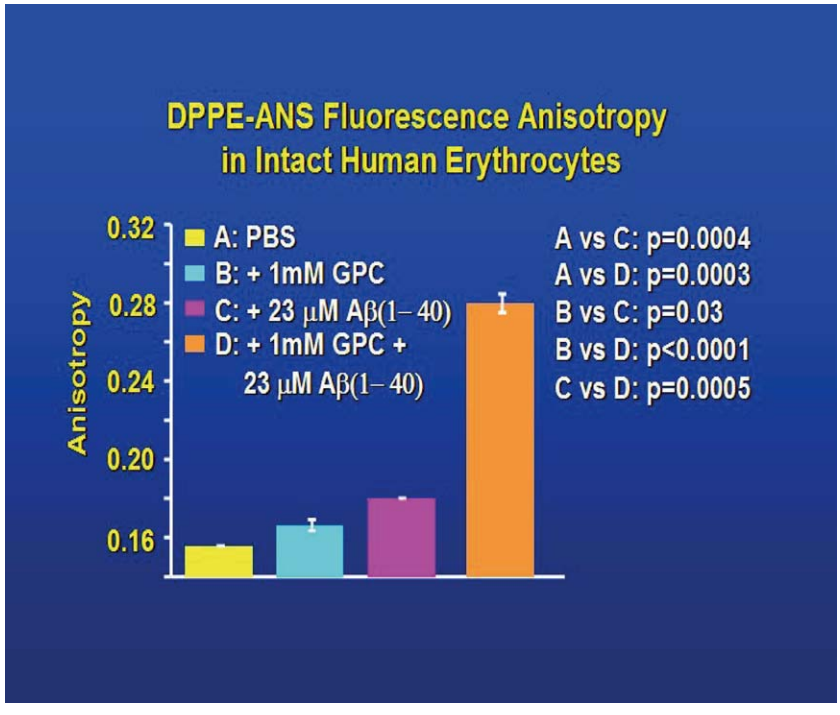

Fig. 2. The effect of GPC, Aβ, and GPC + Aβ on the fluorescence anisotropy of DPPE-ANS in intact human erythrocytes.

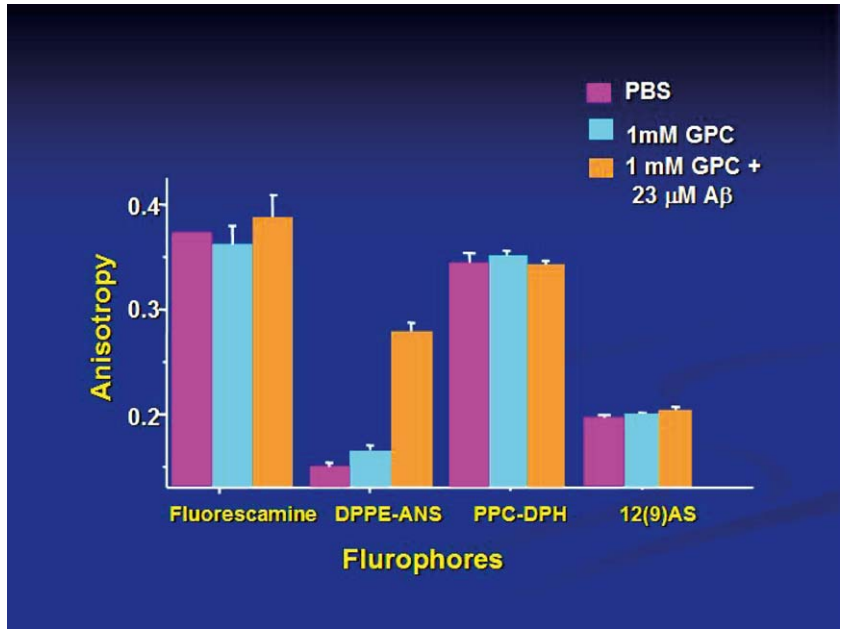

Fig. 3. The effect of GPC, and GPC + Aβ on the fluorescence anisotropy of fluorescamine, DPPE-ANS, PPC-DPH and 12(9)AS in intact human erythrocytes. DPPE-ANS was the only fluorophore that demonstrated significant changes in fluorescence with intact human erythrocytes (PBS vs PBS + GPC + Aβ, $p = 0.0003$ and PBS + GPC vs PBS + GPC + Aβ, $p < 0.0001$).

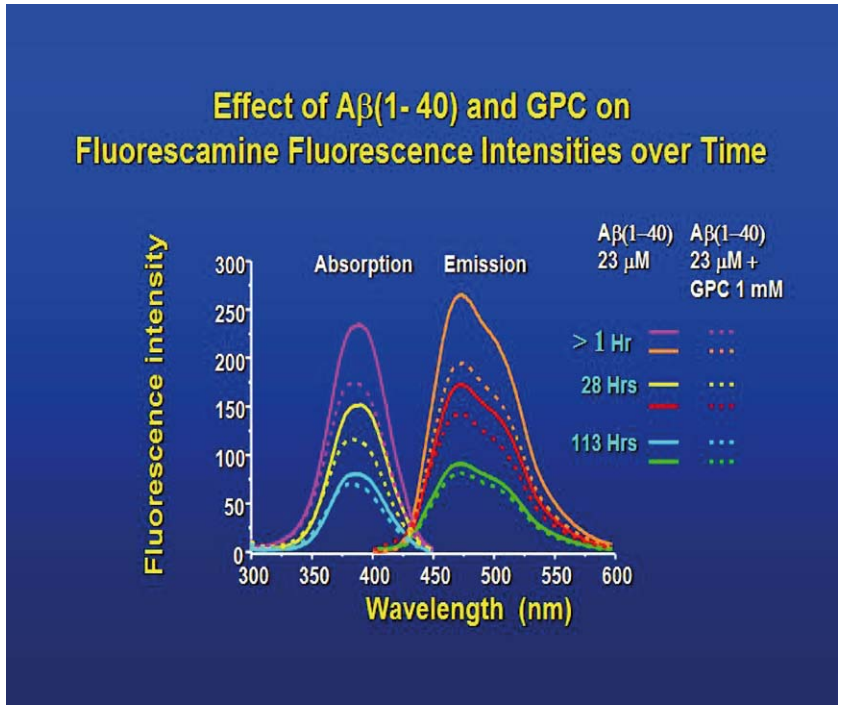

Fig. 4. Fluorescence intensities of fluorescamine-labeled Aβ in the presence and absence of GPC over time. Absorption and emission curves taken at less than 1 h, 28 h, and 113 h are represented by colored lines (red, blue, green, respectively). Curves with dotted lines represent experiments done in the absence of GPC.

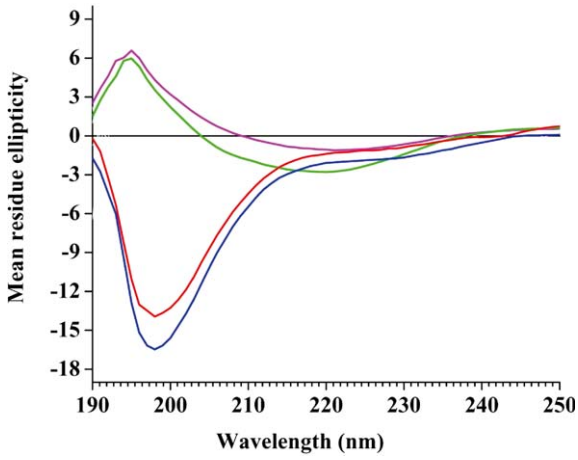

Fig. 5. CD spectra of Aβ in the presence or absence of GPC at two time points, 1–2 h and 10 days. Aβ only (blue curve, 1 h), Aβ + GPC (red curve, 2 h), Aβ only (magenta curve, 10 days), Aβ + GPC (green curve, 10 days).

DISCUSSION

Intact human erythrocytes provide an excellent cellular system in which to investigate the effects of exogenously added molecules on membrane structure, dynamics and function. Our erythrocyte fluorescence experiments were carried out with diluted whole blood (5 μl whole blood is diluted to 4000 μl with PBS buffer, a 800% dilution of whole blood). Previous experiments (16) determined that the procedure used in the present study isolates erythrocytes ($\sim 6.0 \times 10^6$/cc) without damaging the erythrocyte membrane which can occur with repeated washing due to removal of erythrocyte membrane glycolipids and glycoproteins. The removal of these cell membrane constituents was determined by fluorescamine labeling of unwashed erythrocytes and washed erythrocytes in which the washings were examined for fluorescamine fluorescence. These studies demonstrated that PBS washing (centrifugation) removed fluorescence labeled proteins from the erythrocyte surface. Furthermore, ^{31}P NMR analysis demonstrates that erythrocyte labeled with the fluorescamine used in this study does not alter erythrocyte high-energy phosphate metabolism or cellular membrane permeability (20). Thus, intact human erythrocytes provide an easily accessible cellular model membrane system for investigating membrane interactions of Aβ, GPC, and Aβ–GPC complexes.

Several recent studies have shown that Aβ interacts with membranes (21,22). However, to best of our knowledge this is the first study that systematically examined under minimally or non-perturbing conditions, the interactions of GPC or Aβ + GPC on three different erythrocytes regions (membrane surface monitored by fluorescamine, head-group region monitored by DPPE-ANS and hydrocarbon core monitored by PPC-DPH and 12(9)AS). As demonstrated by Fig. 3 only the phospholipid head-group region (DPPE-ANS) is altered by GPC or Aβ + GPC. The erythrocyte membrane surface and hydrocarbon core remain unperturbed. Our study therefore demonstrates that both Aβ and GPC interact predominantly with the erythrocyte head-group region with significant alterations of the molecular dynamics of that region.

The DPPE-ANS fluorophore inserts into the phospholipid head-group region of membranes (16,20). The mobility of the phospholipid head group region of the membrane is reflected by DPPE-ANS anisotropy which is inversely proportional to molecular mobility (23). The increase in DPPE-ANS anisotropy in intact human erythrocytes with Aβ addition reflects insertion of Aβ into the phospholipid head group region of the membrane, decreasing molecular mobility. The pronounced further increase of DPPE-ANS anisotropy in the presence of Aβ plus GPC suggests that GPC is interacting with Aβ forming an Aβ–GPC complex and the complex inserts into the head-group region of the membrane resulting in additional restricted movement of DPPE. Our finding of reduced mobility of DPPE-ANS due to Aβ is consistent with published results of reduced mobility due to Aβ deposition using a different fluorophore, 1,6 diphenylhexa-1,2,3-triene (DPH) (24,25).

The more negative ellipticity of the CD spectrum of Aβ + GPC compared with Aβ alone indicates the addition of GPC results in more β-sheet secondary structure for Aβ in the presence of GPC. We have studied the interaction of Aβ peptide with GPC by two dimensional NMR and ^{31}P NMR spectroscopy. In brief, we have studied by heteronuclear single quantum coherence (HSQC) spectroscopy of ^{15}N labeled Aβ(1–40) peptide and monitor all the amide peaks with time (data not shown). It is observed that due to Aβ interaction with GPC, there is a structural change (evident from intensity and chemical shift change of amide peaks of Aβ peptide) which progresses with time and the Aβ amide peak intensity diminished along with forma-

tion of a precipitate in the bottom of the sample tube.

In our earlier work, aggregation of Aβ was measured by a light scattering method (absorption at 400 nm) (15). This methodology is based on measuring the turbidity due to insoluble, aggregated Aβ (26). Increased Aβ deposition was demonstrated with increased GPC concentration. The binding of fluorescamine to soluble Aβ reported here provides a more sensitive method to detect soluble Aβ and the time-dependent conversion of soluble Aβ into aggregated Aβ. This is an important new method to monitor the conversion of soluble Aβ into fibrillar Aβ. Other fluorescence methods have focused on aggregated Aβ. LeVine (27) reported that thioflavine T (ThT) dye serves as a biological marker (28) to identify Aβ deposition. ThT binds to aggregated, fibrillar Aβ; however, ThT does not bind to the soluble form of Aβ. Binding of ThT to fibrillar Aβ increases the excitation profile of ThT from 385 nm (excitation) and 445 nm (emission) of the free dye to 450 nm (excitation) and 482 nm (emission) of the Aβ bound dye. In summary, Aβ and GPC both interact with the head-group region of the erythrocyte membrane.

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REFERENCES

1. Fratiglioni, L., Launer, L. J., Andersen, K., Breteler, M. M., Copeland, J. R., Dartigues, J. F., Lobo, A., Martinez-Lage, J., Soininen, H., and Hofman, A. 2000. Incidence of dementia and major subtypes in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. Neurology. 54:S10–S15.
2. Yamaguchi, H., Maat-Schieman, M. L., Van Duinen, S. G., Prins, F. A., Neeskens, P., Natte, R., and Roos, R. A. 2000. Amyloid beta protein (Abeta) starts to deposit as plasma membrane-bound form in diffuse plaques of brains from hereditary cerebral hemorrhage with amyloidosis-Dutch type, Alzheimer disease and nondemented aged subjects. J. Neuropathol. Exp. Neurol. 59:723–732.
3. Pike, C. J., Walencewicz, A. J., Glabe, C. G., and Cotman, C. W. 1991. Aggregation-related toxicity of synthetic beta-amyloid protein in hippocampal cultures. Eur. J. Pharmacol. 207:367–368.
4. McLaurin, J. and Chakrabartty, A. 1996. Membrane disruption by Alzheimer beta-amyloid peptides mediated through specific binding to either phospholipids or gangliosides. Implications for neurotoxicity. J. Biol. Chem. 96:26482–26489.
5. Avdulov, N. A., Chochina, S. V., Igbavboa, U., O'Hare, E. O., Schroeder, F., Cleary, J. P., and Wood, W. G. 1997. Amyloid beta-peptides increase annular and bulk fluidity and induce lipid peroxidation in brain synaptic plasma membranes. J. Neurochem. 97:2086–2091.
6. Pettegrew, J. W., Minshew, N. J., Cohen, M. M., Kopp, S. J., and Glonek, T. 1984. P-31 NMR changes in Alzheimer's and Huntington's disease brain. Neurology 34(Suppl 1):281.
7. Pettegrew, J. W., Kopp, S. J., Minshew, N. J., Glonek, T., Feliksik, J. M., Tow, J. P., and Cohen, M. M. 1987. ^{31}P nuclear magnetic resonance studies of phosphoglyceride metabolism in developing and degenerating brain: Preliminary observations. J. Neuropathol. Exp. Neurol. 46:419–430.
8. Pettegrew, J. W., Moossy, J., Withers, G., McKeag, D., and Panchalingam, K. 1988. ^{31}P nuclear magnetic resonance study of the brain in Alzheimer's disease. J. Neuropathol. Exp. Neurol. 47:235–248.
9. Pettegrew, J. W., Panchalingam, K., Moossy, J., Martinez, J., Rao, G., and Boller, F. 1988. Correlation of phosphorus-31 magnetic resonance spectroscopy and morphologic findings in Alzheimer's disease. Arch. Neurol. 45:1093–1096.
10. Barany, M., Chang, Y. -C., Arus, C., Rustan, T., and Frey, W. H. 1985. Increased glycerol-3-phosphorylcholine in post-mortem Alzheimer's brain (letter). Lancet 1:517.
11. Smith, C. D., Gallenstein, L. G., Layton, W. J., Kryscio, R. J., and Markesbery, W. R. 1993. ^{31}P magnetic resonance spectroscopy in Alzheimer's and Pick's disease. Neurobiol. Aging 14:85–92.
12. Pettegrew, J. W., Panchalingam, K., Hamilton, R. L., and McClure, R. J. 2001. Brain membrane phospholipid alterations in Alzheimer's disease. Neurochem. Res. 26:771–782.
13. Pettegrew, J. W., Panchalingam, K., Klunk, W. E., McClure, R. J., and Muenz, L. R. 1994. Alterations of cerebral metabolism in probable Alzheimer's disease: A preliminary study. Neurobiol. Aging 15:117–132.
14. Nitsch, R. M., Blusztajn, J. K., Pittas, A. G., Slack, B. E., Growdon, J. H., and Wurtman, R. J. 1992. Evidence for a membrane defect in Alzheimer disease brain. Proc. Natl. Acad. Sci. USA 89:1671–1675.
15. Klunk, W. E., Xu, C. J., McClure, R. J., Panchalingam, K., Stanley, J. A., and Pettegrew, J. W. 1997. Aggregation of beta-amyloid peptide is promoted by membrane phospholipid metabolites elevated in Alzheimer's disease brain. J. Neurochem. 69:266–272.
16. Pettegrew, J. W., Minshew, N. J., Spiker, D., Tretta, M., Strychor, S., McKeag, D., Munez, L. R., Miller, G. M., Carbone, D., and McClure, R. J. 1993. Alterations in membrane molecular dynamics in erythrocytes of patients with affective illness. Depression 1:88–100.
17. Lobley, A. and Wallace, B. A. 2001. DICHROWEB: A website for the analysis of protein secondary structure from circular dichroism spectra. Biophys. J. 80:373a.
18. Lobley, A., Whitmore, L., and Wallace, B. A. 2002. DICHROWEB: An interactive website for the analysis of protein secondary structure from circular dichroism spectra. Bioinformatics 18:211–212.
19. Wallace, B. A., Janes, R. W., and Orry, A. 2001. Secondary structure information content on the VUV region of synchrotron radiation circular dichroism (SRCD) spectra. Biophys. J. 80:28a.
20. Pettegrew, J. W., Minshew, N. J., and Nichols, J. S. 1982. Fluorescence probes specific for the plasma membrane of intact human erythrocytes and lymphocytes. Life Sci. 30:1101–1105.
21. Mattson, M. P. and Sherman, M. 2003. Perturbed signal transduction in neurodegenerative disorders involving aberrant protein aggregation. Neuromol. Med. 4:109–131.

22. Jayakumar, R., Kusiak, J. W., Chrest, F. J., Demehin, A. A., Murali, J. Wersto, R. P., Nagababu, E., Ravi, L., and Rifkind, J. M. 2003. Red cell perturbations by amyloid beta-protein. Biochim. Biophys. Acta. 1622:20–28.
23. Muller, W. E., Koch, S., Eckert, A., Hartmann, H., and Scheuer, K. 1995. beta-Amyloid peptide decreases membrane fluidity. Brain Res. 674:133–136.
24. Chauhan, V. P., Chauhan, A., and Wegiel, J. 2001. Fibrillar amyloid beta-protein forms a membrane-like hydrophobic domain. Neuroreport 12:587–590.
25. Muller, W. E., Kirsch, C., and Eckert, G. P. 2001. Membrane-disordering effects of beta-amyloid peptides. Biochem. Soc. Trans. 29:617–623.
26. Jarrett, J. T. and Lansbury, P. T., Jr. 1992. Amyloid fibril formation requires a chemically discriminating nucleation event: Studies of an amyloidogenic sequence from the bacterial protein OsmB. Biochemistry 31:12345–12352.
27. LeVine, H. 1993. Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: Detection of amyloid aggregation in solution. Protein Sci. 2:404–410.
28. Kakio, A., Nishimoto, S., Yanagisawa, K., Kozutsumi, Y., and Matsuzaki, K. 2002. Interactions of amyloid beta-protein with various gangliosides in raft-like membranes: Importance of GM1 ganglioside-bound form as an endogenous seed for Alzheimer amyloid. Biochemistry 41:7385–7390.