SCIENTIFIC REPORT

Immobilized amyloid-β protein experiments using SPR technology

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Recent work has demonstrated the utility of biosensor technology for studies of amyloid -β (Aβ) protein. This aggregation-prone peptide, which consists of a 40-42 residue proteolytic fragment of the amyloid precursor protein, is implicated in the progression of Alzheimer's disease. For applications in which protein-protein interactions must be minimized, immobilization of the target as a monomer or aggregate can be used to investigate the aggregation process and to identify inhibitors of this process.

Additionally, the sensitivity of current Biacore instrumentation has allowed for the observation of small molecule binding to Aβ at low ligand concentrations. Methods using immobilized Aβ allow for controlled studies of protein-Aβ, Aβ -Aβ, and inhibitor-Aβ interactions.

Protein aggregation appears to be linked to the progression of Alzheimer's disease. (AD), and the presence of amyloid plaques in the brain is an invariant feature of the disease. The primary component of these neuritic plaques is the amyloid-β (Aβ) protein, and its aggregation into plaques has been proposed to be a cause of AD neurodegeneration (Hardy and Higgins, 1992). The amyloid cascade hypothesis continues to be controversial, and the process of Aβ protein aggregation and its biological consequences remains the subject of intense scrutiny (Rochet and Lansbury, 2000). Outstanding questions such as the thermodynamic basis for aggregation, the structure of the aggregates, the role of amyloid binding proteins, and the utility of inhibiting the aggregate's toxic properties continue to drive the development of new methods. Biosensors that use immobilized Aβ for the study of amyloid-protein, amyloid-amyloid, and amyloid-inhibitor interactions have all been reported. Each of these approaches have provided essential data for several aspects of amyloid interactions.

Binding targets that are prone to aggregation in solution present inherent problems for the development of binding assays. Results from solution assays may be difficult to interpret if the aggregation state of the binding target is not constant over the course of the experiment. Additionally, if multiple preparations of the protein are to be used, the aggregation state must remain constant between experiments to maintain reproducibility. Both of these problems can be avoided by immobilization of the aggregating species, either in a disaggregated form or as an aggregate (Table 1). Immobilization maintains site isolation of the aggregating species, and in some cases the surfaces can be regenerated for multiple experiments. The current literature provides instructive examples for the use and construction of amyloid binding assays for identifying Aβ binding proteins, studying Aβ aggregation, and identifying Aβ-binding small molecules.

Abbreviations:

Aβ, amyloid-β protein; AD, Alzheimer's disease; Aha, aminohexanoic acid; CMD, carboxymethyl dextran; DMSO, dimethyl sulfoxide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; R_{eq}, equilibrium response; RU, response units; SPR, surface plasmon resonance.

IDENTIFYING Aβ BINDING PROTEINS

Biacore's SPR technology has been used to study the binding interactions of amyloid with apolipoproteins (Shuvaev and Siest, 1996; Wood *et al*., 1996) and serum albumin (Bohrmann *et al*., 1999). Apolipoprotein isoforms, such as ApoE3, have been found to be a genetic factor in AD. The exact role of apolipoproteins in AD is unclear; however, it has been proposed that these proteins bind to and prevent the aggregation of Aβ. Shuvaev *et al*. immobilized Aβ using amine coupling, and they observed the interaction of several apolipoproteins believed to be relevant in AD pathology (Shuvaev and Siest, 1996). Using this configuration, the affinity and kinetics of binding for ApoE3, ApoA-I, and ApoA-II were determined. The authors used the effects of salt concentration and pH on these interactions to support a predominantly hydrophobic model of binding.

A later study by Wood *et al*. examined Apo-E3-Aβ interactions using immobilized Aβ

Reference	β-Amyloid used	Aggregation state	Immobilization Chemistry	Sensor chip used	Immobilization level (RU)	Purpose
Shuvaev and Siest, 1996	Aβ (1-40)	NR	amine coupling	CM5	7200	apolipoprotein binding
Wood et al., 1996	Aβ (1-40)	aggregated	amine coupling	CM5	NR	apolipoprotein binding
Bohrmann et al., 1999 Bohrmann et al., 2000	Aβ (1-42) Aβ (1-42)-biotin	monomer isolated by ultracentrifugation or aggregated	streptavidin-biotin antibody capture	CM5 SA	200-1200	inhibitor binding
Myszka et al., 1999	Aβ (1-40)	aggregated	amine coupling	CM5 C1	1000-3000	Aβ aggregation kinetics
Heal et al., 2002	Aβ (1-40)	NR	amine coupling	CMD surface	3500*	inhibitor binding
Cairo et al., 2002	Aβ (10-35)-Aha-C	dissaggregated with DMSO	thiol coupling	CM5 B1	1400	inhibitor binding
Hasegawa et al., 2002	Aβ (1-40)	aggregated	amine coupling	F1	900-3800	Aβ aggregation kinetics

Table 1. Summary of immobilization strategies reported in the literature. *Immobilization was reported in ng/mm^2 and converted to RU. Abbreviations: CM5 - standard carboxymethyldextran (CMD) surface; B1 - CMD surface with less negative charge than CM5; F1 - CMD with shortened dextran matrix; SA - immobilized streptavidin surface; C1 - Carboxylated surface with no dextran.
NR - not reported.

aggregates. The authors found that aggregated Aβ, but not monomeric Aβ could bind to the immobilized aggregate. Additionally, the apolipoprotein, ApoE3, bound specifically to this surface (Wood *et al*., 1996). The interaction of ApoE3 with Aβ has been supported by immunoprecipitation studies (Zhou *et al*., 2002). Further studies of Aβ showed that its aggregation in solution was inhibited in the presence of ApoE3. These results suggest that the ApoE3 isoform may act as a physiologic agent for controlling amyloid aggregation.

Aβ AGGREGATION

The immobilization of Aβ on a sensor chip allows direct measurement of aggregation under controlled conditions. The physical separation of the immobilized component can prevent interactions between aggregates. Therefore, this strategy can allow for experiments that investigate the consequences of addition of a purified solution component, such as monomeric peptide, without interference from aggregate-aggregate interactions. Importantly, SPR measurements of fibril extension at a surface do not require the incorporation of extrinsic dyes or reporter groups. Myszka *et al*. established this method-ology using Aβ (1-40) aggregates immobilized using amine coupling (Myszka *et al*., 1999). Solutions containing Aβ (1-40) showed rapid association of the peptide with the surface and apparent biphasic dissociation. Although these responses were highly reproducible, they could vary based on the aggregation state of the peptide in solution. Myszka *et al*. did not propose a detailed model from their observations of Aβ aggregation kinetics; however, they did establish that the dissociation kinetics of the interaction are more complex than would be expected for a single rate of dissociation. They propose that these data are consistent with a dock-and-lock mechanism of Aβ fibril formation previously proposed by Esler *et al*. (Esler *et al*., 2000).

Hasegawa *et al*. have recently reported a more detailed kinetic study of Aβ aggregation. Their data confirm the results of Myszka *et al*. and allow them to propose a more detailed kinetic model (Hasegawa *et al*., 2002). Using a similar experimental configuration to Myszka *et al*., aggregated Aβ was immobilized and the binding of disaggregated Aβ was observed.

The immobilization of fibrillar amyloid aggregates was confirmed by AFM imaging of the biosensor surface. These images support the relative site isolation of immobilized fibrils. Examination of the surface after exposure to soluble Aβ supports that the binding of Aβ to the surface results in fibril extension. The kinetics of dissociation of Aβ from the surface were complex, and were accurately described by the use of a first order kinetic model that included an additional exponential decay term. This second-order dissociation phase is consistent with earlier proposals of a dock-and-lock mechanism. These results are similar to the decay constants observed using radioisotope-labeled Aβ to monitor the kinetics of monomer deposition (Esler *et al*., 2000; Hasegawa *et al*., 2002).

IDENTIFYING Aβ-BINDING SMALL MOLECULES

The precise role of Aβ in the progression of AD remains an open question (Selkoe, 2001). Small molecule inhibitors of amyloid toxicity and aggregation can be valuable tools to study this

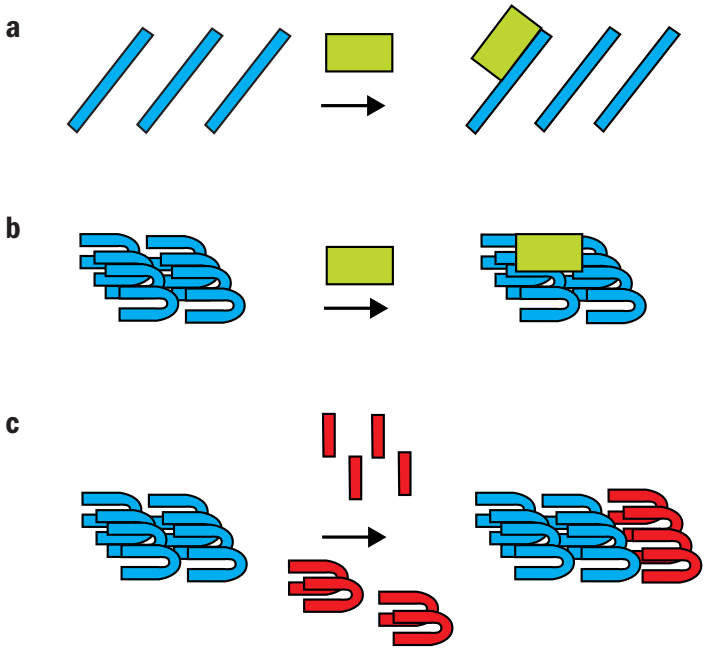

Figure 1. Configurations for immobilized Aβ binding assays.
(a) Immobilization of monomeric protein to observe small molecule and polypeptide binding.
(b) Immobilization of protein aggregates to observe small molecule and polypeptide binding.
(c) Immobilization of aggregates to observe the binding of monomeric or aggregated species to the surface.
Boxes represent small molecule or protein ligands for Aβ, and rods represent Aβ protein.

process and its consequences (Findeis, 2000). Additionally, if the amyloid cascade hypothesis holds, such inhibitors could lead to effective treatments for the disease. Although numerous methods have been used to evaluate candidate inhibitors, SPR has only recently been used to directly measure the binding of potential inhibitors to Aβ. The sensitivity of current SPR instrumentation allows for examination of the binding interactions of both small molecules and polypeptides.

The first published example of an SPR assay that directly showed small molecule binding to Aβ was reported by Bohrmann *et al*. (Bohrmann *et al*., 2000). These authors immobilized amyloid fibrils to the sensor chip surface and determined the relative binding response for each compound. They observed binding of at least one compound that was also found to be an inhibitor of toxicity. Heal *et al*. also examined the binding of polypeptides to immobilized Aβ (Heal *et al*., 2002). In both of these studies, qualitative comparisons of ligand affinity could be obtained. It is likely that these interactions were difficult to observe due to the low affinity of the interactions and the limited solubility of the ligands.

Both qualitative and quantitative binding data can be obtained using immobilized Aβ in Biacore binding assays. We have used a fragment of the Aβ peptide, Aβ (10-35) for binding studies using peptide inhibitors of amyloid toxicity (Cairo *et al*., 2002). Aβ was dissolved in DMSO and then immobilized via an additional C-terminal cysteine residue for oriented thiol coupling (Johnsson *et al*., 1991). In these studies, affinities were determined by plotting the equilibrium responses (R_{eq}) versus the concentration of ligand (Figure 2). The pentapeptide sequences showed only weak affinity to the surface (ca. 1 mM), and these binding data were used for a relative assessment of binding. Alternatively, the binding for peptides with higher affinity for Aβ reached saturation, and the data provided a quantitative measurement of ligand binding (ca. 40 μM). Using this procedure to investigate a series of related peptide sequences, structure-activity relationships for ligand binding to Aβ could be established. These experiments reveal that for peptides in which the central hydrophobic domain of Aβ, Aβ (16-20), is flanked with positively charged residues, higher affinities for Aβ are observed. The location of these charges is essential to improved activity: the inclusion of N-terminal lysine residues has no effect, while C-terminal lysine residues dramatically enhance binding. Importantly, ligands with the tightest affinities were the same ligands that provided the most protection from cellular toxicity of Aβ (Cairo *et al*., 2002; Lowe *et al*., 2001).

CONCLUSIONS

Protein aggregates are difficult to study by traditional biochemical techniques. To investigate these molecular species, new tools are required to understand and manipulate protein aggregation processes. Surface immobilization of aggregating proteins provides a means to examine the interactions of relatively homogeneous species. Such experiments avoid aggregation artifacts and conserve material. The sensitivity of current surface detection methods like Biacore's SPR technology, allow for detailed kinetic and thermodynamic studies of both small molecule and protein binding to immobilized species. The use of Aβ in assays that exploit these features of SPR has the

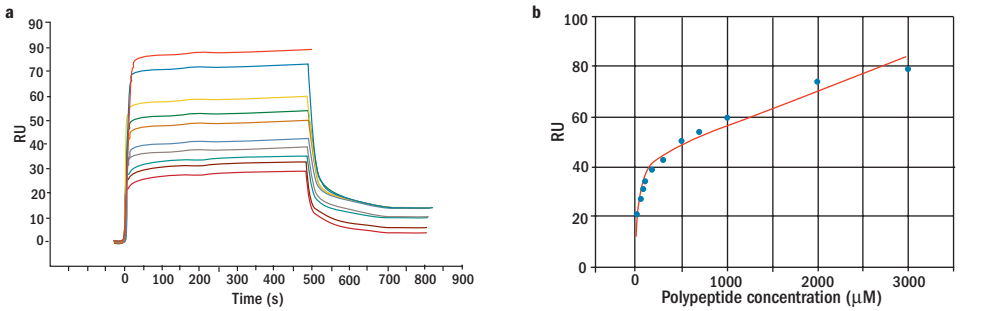

Figure 2. Binding of polypeptides to immobilized Aβ (10-35) on the surface of Pioneer Chip B1. (a) Sensorgrams of KLVFFKKKKKK injections obtained using Biacore® 2000. Concentrations injected are 3000, 2000, 1000, 700, 400, 300, 200, 100, 70, and 50 µM. (b) Plot of R_{eq} vs. concentration. Data were fit using a single site model that includes a non-specific term.
From Cairo *et al.* (2002).

potential to further our understanding of the process of aggregation and the role that native proteins or small molecule inhibitors could play.

One important observation from these reports of Aβ immobilization suggests that distinct aggregation states can be immobilized to a surface. In two different studies, Bohrman *et al.* observed interactions between immobilized Aβ (1-42) and small molecules or albumin only if the immobilized protein was in an aggregated form (Bohrmann *et al.*, 1999; Bohrmann *et al.*, 2000). We found that Congo red binding to disaggregated Aβ on a surface was weaker than that reported earlier for binding to an Aβ aggregate (Cairo *et al.*, 2002; Han *et al.*, 1996). These observations suggest that distinct aggregation states can be immobilized and studied as separate entities. As new intermediates in the aggregation pathway are identified, immobilization strategies may allow studies of their interactions with other aggregates and inhibitors (Koo *et al.*, 1999; Murphy and Pallitto, 2000).

From the reports discussed here, it is clear that Biacore techniques can be used to identify new small molecule inhibitors of amyloid aggregation and toxicity. Current interest has begun to focus on the identification of compounds that specifically bind to Aβ . These molecules could have potential uses in both diagnosis and treatment of the disease. Additionally, methods for identification of Aβ inhibitors may prove generally applicable to other protein aggregation systems that have been difficult to study with traditional methods.

REFERENCES

Bohrmann, B., Tjernberg, L., Kuner, P., Poli, S., Levet-Trafit, B., Naslund, J., Richards, G., Huber, W., Dobeli, H., and Nordstedt, C.
J Biol Chem 274: 15990-5 (1999)

Bohrmann, B., Adrian, M., Dubochet, J., Kuner, P., Muller, F., Huber, W., Nordstedt, C. and Dobeli, H. J.
Struct Biol 130,: 232-46 (2000)

Cairo, C. W., Strzelec, A., Murphy, R. M. and Kiessling, L. L.
Biochemistry 41: 8620-9 (2002)

Esler, W. P., Stimson, E. R., Jennings, J. M., Vinters, H. V., Ghilardi, J. R., Lee, J. P., Mantyh, P. W. and Maggio, J. E.
Biochemistry 39: 6288-95 (2000)

Findeis, M. A.
Biochim Biophys Acta-Molecular Basis of Disease 1502: 76-84 (2000)

Han, H., Cho, C. G. and Lansbury, P. T., Jr.
J Am Chem Soc 118: 4506-7 (1996)

Hardy, J. A. and Higgins, G. A.
Science 256: 184-85 (1992)

Hasegawa, K., Ono, K., Yamada, M. and Naiki, H.
Biochemistry 41: 13489-98 (2002)

Heal, J. R., Roberts, G. W., Christie, G. and Miller, A. D.
Chembiochem 3: 86-92 (2002)

Johnsson, B., Löfås, S. and Lindquist, G.
Anal Biochem 198: 268-77 (1991)

Koo, E. H., Lansbury, P.T., Jr., and Kelly, J. W.
Proc Natl Acad Sci USA 96: 9989-90 (1999)

Lowe, T. L., Strzelec, A., Kiessling, L. L. and Murphy, R. M.
Biochemistry 40: 7882-9 (2001)

Murphy, R. M. and Pallitto, M. R.
J Struct Biol 130: 109-22 (2000)

Myszka, D. G., Wood, S. J., and Biere, A. L.
Methods Enzymol 309: 386-402 (1999)

Rochet, J. C. and Lansbury, P.T., Jr.
Curr Opin Struct Biol 10: 60-8 (2000)

Selkoe, D. J.
Physiol Rev 81: 741-766 (2001)

Shuvaev, V. V. and Siest, G. R.
FEBS Lett 383: 9-12 (1996)

Wood, S. J., Chan, W. and Wetzel, R.
Chem Biol 3: 949-56 (1996)

Zhou, Z. M., Relkin, N., Ghiso, J., Smith, J. D., and Gandy, S.
Molecular Medicine 8: 376-81 (2002)