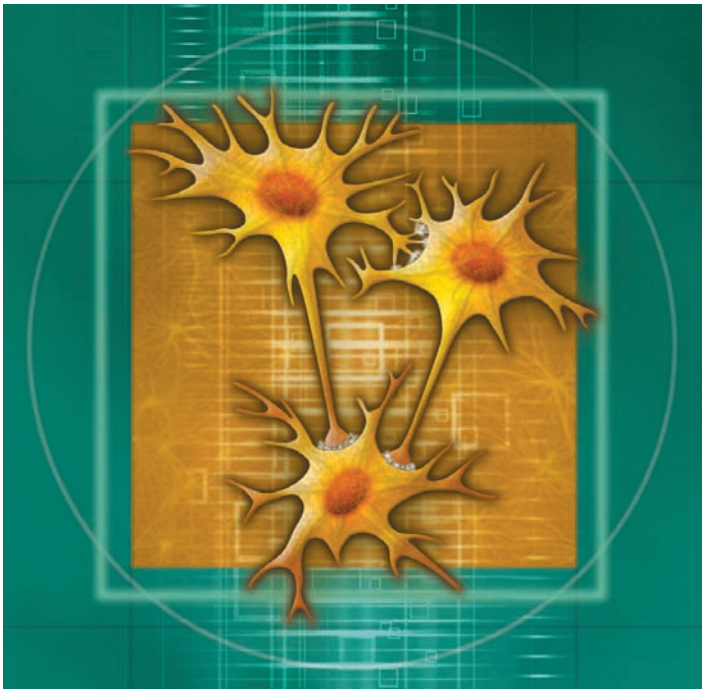
GE Healthcare



Neuroscience

Label-free interaction analysis: revealing the secrets of biomolecular interactions

Biacore

Biacore systems from GE Healthcare

For over 15 years, Biacore™ systems have enabled comprehensive, label-free characterization of binding events, giving unique insights into how proteins and other biomolecules interact with each other, and helping scientists to better understand their biological functions.

The wealth of information provided by Biacore systems, such as binding kinetics, affinity, specificity, thermodynamics and concentration is valuable across many applications and plays a vital role in characterizing the binding properties of a wide range of molecules, from proteins to low molecular weight compounds. A broad portfolio of instruments, reagents and convenience products supports the needs of scientists throughout academic research and pharmaceutical and biotechnology industry workflows. Biacore systems meet the high demands for quality, performance and regulatory compliance throughout research, drug discovery, development, manufacturing and quality control.

For more information about Biacore systems and applications visit www.biacore.com.

» Did you know that.....

A database of research papers describing applications using label-free interaction analysis is continually updated and presented on www.biacore.com.

Label-free interaction analysis using Biacore systems in neuroscience

Biacore assays are now firmly established in neurological research. This booklet reviews some recent papers from both clinical laboratories in which Biacore systems are used to detect neurological disease markers in complex biological matrices and basic research facilities working to deconstruct the molecular mechanisms behind neuropathies.

Alzheimer's disease

Searching for inhibitors of β-amyloid toxicity

- Identify peptides that bind Aβ and inhibit neurotoxicity in Alzheimer's disease
- Avoid problems of target aggregation seen in solution based assays: Immobilization of the target on a sensor surface allows the user to control density and orientation
- Use real time polymerization on a sensor surface to understand why Aβ forms aggregates exclusively in tissues of the central nervous system
- Identify targets for screening mimetics of neuron survival factors
- Identify cofactors involved in protein interactions

Identifying small peptide inhibitors of neurotoxic proteins

β-amyloid (Aβ) aggregates are present in neurotoxic lesions in the brain of Alzheimer's disease patients. The rationale for a Biacore study by Laura Kiessling and colleagues at the University of Wisconsin in Madison (1) was to identify peptides that tightly bind and inhibit the toxic neurodegenerative effects of Aβ. Short peptides directed to the self-recognition motifs in Aβ were synthesized and label-free protein interaction analysis was used to screen the affinities of the interactions.

Solution-based methods for screening Aβ inhibitors consume large amounts of the target and they are limited by the tendency of Aβ to aggregate in solution and thus present multiple target forms. This problem is avoided in Biacore assays because the target is immobilized on a sensor surface at a density and in an orientation that can be carefully controlled by the user. A 26-amino acid fragment of Aβ with a C-terminal cysteine residue was immobilized at low density on a sensor surface and thus presented a uniform surface. Inter-assay variability was low as it was possible to regenerate and reuse a single sensor surface many times. A titration series of each peptide, based on variations of a five amino acid sequence corresponding to the central hydrophobic domain of Aβ known to be responsible for self association was injected over the prepared surface and the response was recorded.

A pattern emerged in which affinity for Aβ was sensitive to site-specific positioning of positively charged lysine residues. Peptides in which three of these residues were separated from the basic sequence by three negatively charged residues had a 14-fold lower affinity for Aβ, while the affinity of a peptide of similar size bearing three directly adjacent lysine residues was almost unchanged.

The peptides were then tested for cytotoxicity in microtiter assays by incubating Aβ with neuroblastoma cells in the presence of peptides. Compounds that bound Aβ were efficient protectors against toxicity in viability assays while the ability to protect gradually diminished with reducing affinity (Table 1). It is hoped that these peptides may be useful as probes in investigations of the mechanisms underlying amyloid plaque formation and in the design of drugs in the treatment of Alzheimer's disease.

Table 1. Affinity of peptides from the central hydrophobic domain of Aβ for the full protein

Peptide sequence[1]	K_D (mM)	Viability (%)[2]
KLVFFRRRRRR	40	> 90
KLVFFKKKKKK	40	80–90
KLVWWKKKKKK	40	80–90
KLVFFKKKK	37	70–80
KLVFWKKKKKK	65	70–80
KLVFFKK	80	70–80
KLVFFKKKEEE	90	60–70[3]
KKKKLVFF	180	60–70[3]
KLVFFEKEKEK	300	60–70[3]
KKKKKK	400	60–70[3]
KLVFFEEEKKK	1300	60–70[3]

[1] K=lysine, L=leucine, V=valine, F=phenylalanine, R=arginine, W=tryptophan, E=glutamic acid.

[2] Cellular viability of human neuroblastoma cells was assessed in a microtiter assay using an MTT assay in which cells were exposed to Ab in the presence or absence of peptides.

[3] This is a similar level of viability seen after treatment with negative controls.

Albumin: a natural inhibitor of Aβ polymerization?

Although Aβ is expressed by almost all nucleated cells, it is not known why the protein forms lesions exclusively in tissues of the central nervous system. The most common plasma proteins are also present in cerebrospinal fluid (CSF), but at much lower concentrations. One study reported the use of a Biacore system in a screening strategy to find out if any proteins common to both plasma and CSF could bind Aβ and thus potentially inhibit deposition (2).

Plasma and CSF proteins were tested for their capacity to inhibit Aβ polymerization where Aβ was immobilized as a template to which further Aβ was added in the presence of the test protein. This revealed that the substances that most effectively inhibited polymerization were albumin, α1-antitrypsin, IgA and IgG, proteins found only at low concentrations in CSF. The authors suggest that albumin, at its normal concentration in plasma, may bind and thus prevent the deposition of polymerized Aβ.

Inhibitors of apoptosis

The findings of Dr Dave Dawbarn's group at the University of Bristol may help accelerate the search for mimetics of cell survival factors (3). A selective loss of TrkB, a tyrosine kinase receptor for certain neurotrophins, has been reported in Alzheimer's disease. One ligand of TrkB, brain-derived neurotrophic factor (BDNF) is a known neuronal survival factor. Naylor *et al*. used label-free protein interaction analysis to identify the extracellular docking site for neurotrophins on TrkB. Specifically, the role of the Ig-like domain of TrkB was investigated. Recombinant Ig-like domain was immobilized on a sensor surface. A titration series of BDNF was injected over the prepared surfaces and the affinities were calculated. The authors suggest that the Ig-like domain of TrkB is responsible for high-affinity binding of BDNF and that it may thus be used to screen for mimetics in the treatment of neurodegenerative diseases.

In addition to Aβ, serum amyloid P component (SAP) is also found in amyloid deposits and forms aggregates in the presence of calcium. A study by Vorum *et al*. demonstrates how a Biacore system was used to characterize the conditions necessary for the interaction between SAP and calumenin, a protein that helps maintain the structural integrity of SAP (Fig 1 to Fig 3) (4).

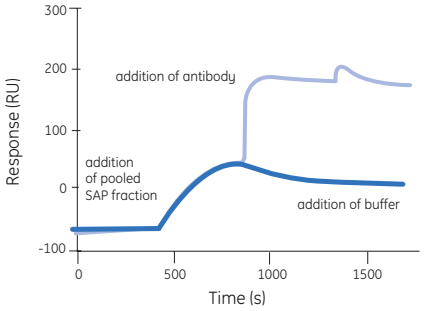


Fig 1. Verification of SAP-binding to immobilized calumenin by the additional binding of anti-SAP antibody.

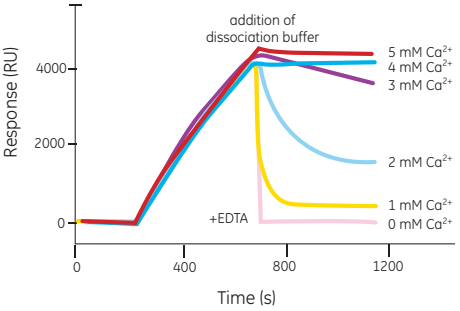


Fig 3. After formation of the calumenin: SAP complex, Ca^{2+} is necessary to maintain the complex as shown by the effect of exclusion of Ca^{2+} from the dissociation buffer on the dissociation rate.

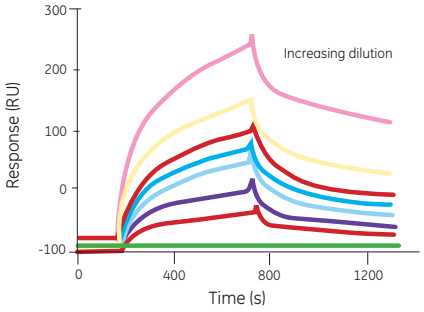


Fig 2. Binding of SAP to immobilized calumenin.

Calumenin was immobilized on a sensor surface and SAP was injected over the prepared surface. The results showed that the formation and stability of the complex required the presence of calcium. Given the tendency of both SAP and calumenin to form insoluble complexes, calumenin may participate in amyloidosis, the pathological process by which Aβ is deposited in the CNS in patients with Alzheimer's disease.

References

1. Cairo, C. W. *et al.*
   Affinity-based inhibition of beta-amyloid toxicity.
   Biochemistry **41**, 8620–8629 (2002).
2. Bohrmann, B. *et al.*
   Endogenous proteins controlling amyloid beta-peptide polymerization. Possible implications for beta-amyloid formation in the central nervous system and in peripheral tissues.
   J. Biol. Chem. **274**, 15990–15995 (1999).
3. Naylor, R. L. *et al.*
   A discrete domain of the human TrkB receptor defines the binding sites for BDNF and NT-4.
   Biochem. Biophys. Res. Commun. **291**, 501–507 (2002).
4. Vorum, H. *et al.*
   Calumenin interacts with serum amyloid P component.
   FEBS Lett. **465**, 129–134 (2000).

Finding the causes of neurotoxicity caused by Aβ fibril deposition

- Follow peptide polymerization in real time on a sensor surface
- Discover how lipids in neuron plasma membranes are important factors in initiating the accumulation of toxic aggregates of Aβ

In 1999, Myszka *et al*. published results from a Biacore-based, on-chip assay suggesting that Aβ peptides aggregate to form toxic fibrils by a "lock and dock" mechanism (5). Since then, Hasegawa *et al*. at Fukui Medical University have used a Biacore system in a kinetic and thermodynamic analysis of Aβ aggregation (6). Their experiments were designed to mimic the polymerization of Aβ fibrils that occurs prior to, or during their deposition in toxic plaques in the brain of Alzheimer's disease patients.

Hasegawa *et al*. prepared Aβ fibrils allowing a solution of Aβ to spontaneously aggregate. The preformed fibrils were then sonicated and monomeric Aβ was added. After a further incubation for 24 hours, the preparation was immobilized on a sensor surface. The immobilized fibrils functioned as "seeds" from which extensions were able to grow. This process of elongation was then monitored in real time.

Association of monomeric Aβ with the growing polymer was characterized by a linear increase in signal during the course of the injection phase. That the reaction rate increased linearly with both ligand density and analyte concentration is consistent with a first order kinetic model of an extension reaction and reflects consecutive association of monomeric Aβ onto the ends of a growing polymer. Further, dissociation was biphasic, initially showing fast dissociation followed by a slower linear phase. The authors suggest that these fast and slow phases may reflect reverse reactions of the dock and lock phases of fibril elongation, respectively (see (5) for the hypothesis of lock and dock elongation dynamics). The kinetics of the linear slow phase were also consistent with a first-order kinetic model, indicating that the slow phase represents the consecutive dissociation of the "locked" Aβ from the ends of fibrils.

Although the neurotoxicity of aggregated Aβ is well established, it is not known how it exerts its degenerative effect. Kremer and Murphy (2003) at the University of Wisconsin-Madison recently developed a Biacore-based assay to reveal how aggregation and lipid composition may regulate the interaction of Aβ with neuronal plasma membranes (7). Specifically constituted liposomes were captured on a sensor surface designed to capture lipid membrane vesicles while allowing them to maintain a plasma membrane-like, lipid bilayer structure.

More Aβ bound to anionic (POPG) than to zwitterionic (POPC) liposomes. After a similar dissociation time, approximately 50% of the Aβ was lost from POPC liposomes, leaving a bound fraction as a potential template for polymerization. The association between aged (aggregated) Aβ and liposomes was much slower and did not approach equilibrium, although very little of the bound, aggregated Aβ dissociated during the washing phase. These results were mirrored in experiments in which ^{125}I-labelled monomeric or aged Aβ were separated from solutions using liposomes bound to magnetic beads.

In a similar vein, the group of David Small at the University of Melbourne used a Biacore system to define how membrane-bound cholesterol regulates the interaction of Aβ with neurons (8). To do this, they immobilized synthetic lipid bilayers (SUV) or membrane-enriched preparations from vascular smooth muscle cells (SMC) on a sensor surface and Aβ was then injected over this surface. The effect of ionic strength on the interaction between Aβ and the SUV and SMC preparations was tested by varying the concentration of NaCl in the running buffer.

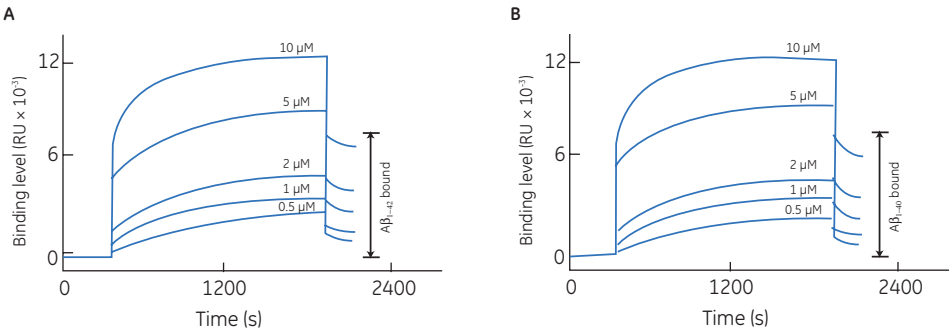


Fig 4. Interaction profiles of (A) non-aged $A\beta_{1-42}$ and (B) $A\beta_{1-40}$ to SUV containing cholesterol and phospholipids. The interaction profiles indicate how the amount of bound peptide was calculated (the difference between the binding level (RU value) recorded 20 seconds after the start of peptide dissociation and the RU value recorded just before adding the peptide). Figure reproduced, with permission, from Subasinghe *et al.*, *J. Neurochem.* **84**, 471–479 (2003).

Using liposomes constructed of 60% cholesterol and 40% mixed phospholipids, binding of high concentrations of peptides $A\beta_{1-42}$ and $A\beta_{1-40}$ were irreversible, with the interaction profile baselines remaining considerably elevated after dissociation (Fig 4). To help define the physical nature of the interaction, Aβ was allowed to bind to immobilized liposomes in low ionic strength buffer (to maintain the selected aggregation state) and dissociation was tested using buffers containing increasing NaCl concentrations. At higher NaCl concentrations, the rate of dissociation greatly increased (Fig 5), suggesting that Aβ may primarily bind membranes by electrostatic rather than hydrophobic forces and implying that the negative charges on membrane lipid heads are important. This hypothesis of Subasinghe *et al.* was strengthened by data from experiments showing that by using succinylated Aβ as the binding partner in solution (a process by which positive amino acids in a peptide are neutralized), binding was virtually abolished (Fig 6). Additionally, succinylated Aβ was shown to be less cytotoxic in cell-based assays.

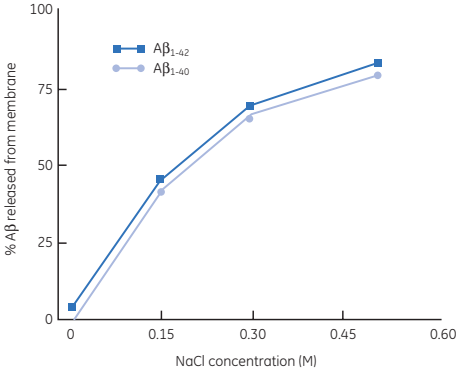


Fig 5. Effect of NaCl on the dissociation of $A\beta_{1-40}$ (spheres) and $A\beta_{1-42}$ (squares) from synthetic lipid membranes. $A\beta_{1-40}$ and $A\beta_{1-42}$ were dissolved in phosphate buffer without NaCl and immediately injected over a cholesterol: phospholipid surface. Twenty minutes after addition of either peptide, phosphate buffer containing various concentrations of NaCl was injected. The percentage of peptide released from the membrane at the end of this period was then determined. Figure reproduced, with permission, from Subasinghe *et al.*, *J. Neurochem.* **84**, 471–479 (2003).

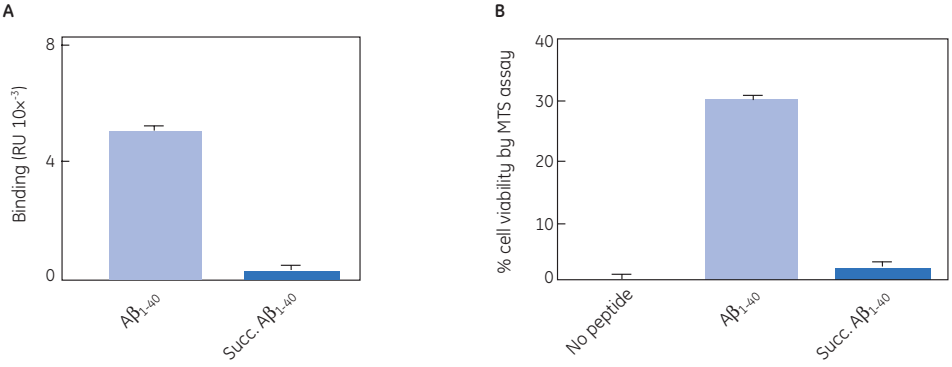


Fig 6. Binding of (A) $A\beta_{1-40}$ and succinyl-$A\beta_{1-40}$ to SUV containing cholesterol and phospholipids, and (B) the effect of $A\beta_{1-40}$ and succinyl-$A\beta_{1-40}$ on vascular SMC viability. Figure reproduced, with permission, from Subasinghe *et al.*, *J. Neurochem.* **84**, 471–479 (2003).

Aβ also bound to immobilized SMC membrane-enriched preparations. However, if cholesterol synthesis by cultured cells was inhibited by treatment with lovastatin prior to harvest and the preparation of membranes, Aβ binding was reduced to about 10% of the binding level using membranes prepared from untreated cells. Further, lovastatin-treated cells were more resistant to Aβ toxicity in cell-based assays.

In this comprehensive body of work, Subasinghe *et al*. used a combination of cell-based assays and label-free interaction analysis to confirm the importance of membrane binding for Aβ cytotoxicity and to show that the interaction is largely dependent on electrostatic forces. This information may thus enable the development of novel ways to inhibit the formation of neurotoxic lesions and arrest the progress of Alzheimer's disease.

References

5. Myszka, D. G. *et al.*
   Analysis of fibril elongation using surface plasmon resonance biosensors
   Methods Enzymol. **309**, 386–402 (1999).
6. Hasegawa, K. *et al.*
   Kinetic modeling and determination of reaction constants of Alzheimer's β-amyloid fibril extension and dissociation using surface plasmon resonance.
   Biochemistry **41**, 13489–13498 (2002).
7. Kremer, J. J. and Murphy, R. M.
   Kinetics of adsorption of β-amyloid peptide Aβ(1–40) to lipid bilayers.
   J. Biochem. Biophys. Methods. **57**, 159–169 (2003).
8. Subasinghe, S. *et al.*
   Cholesterol is necessary both for the toxic effect of Aβ peptides on vascular smooth muscle cells and for Aβ binding to vascular smooth muscle cell membranes.
   J. Neurochem. **84**, 471–479 (2003).

Spinal muscular atrophy

Searching for the molecular background to motor neuron death

- Use label-free interaction analysis in sequential binding experiments as an alternative to immunoprecipitation
- Reconstruct sequential interactions on a sensor surface to understand the molecular mechanisms underlying protein function

Biacore assays: a rapid alternative to immunoprecipitation

> "It is straightforward and significantly faster than the standard technique."
>
> *Dr Christian Lorson, Arizona State University*

Spinal muscular atrophy (SMA) is predominantly a childhood disease of motor neurons in the spinal cord. The disease is strongly linked to mutations in the SMN1 (survival motor neuron 1) gene. The work of Philip Young and colleagues at Arizona State University suggests that the clinical symptoms of the disease may be due to increased motor neuron death through the inability of mutated SMN protein to dimerize, and thereby sequester and inhibit the pro-apoptotic function of the tumor suppressor protein, p53 (9).

Sequential binding experiments demonstrated how label-free protein interaction analysis may be used as an alternative to immunoprecipitation and may be better for detecting molecular interactions in whole cell extracts (Fig 7). Briefly, anti-p53 antibody was immobilized on a sensor surface. Next, whole cell extracts from SMN-transfected cells were injected over the prepared surface, followed by an anti-SMN antibody. Cell extracts that did not contain detectable levels of p53 were used in parallel and demonstrated the specificity of the interaction.

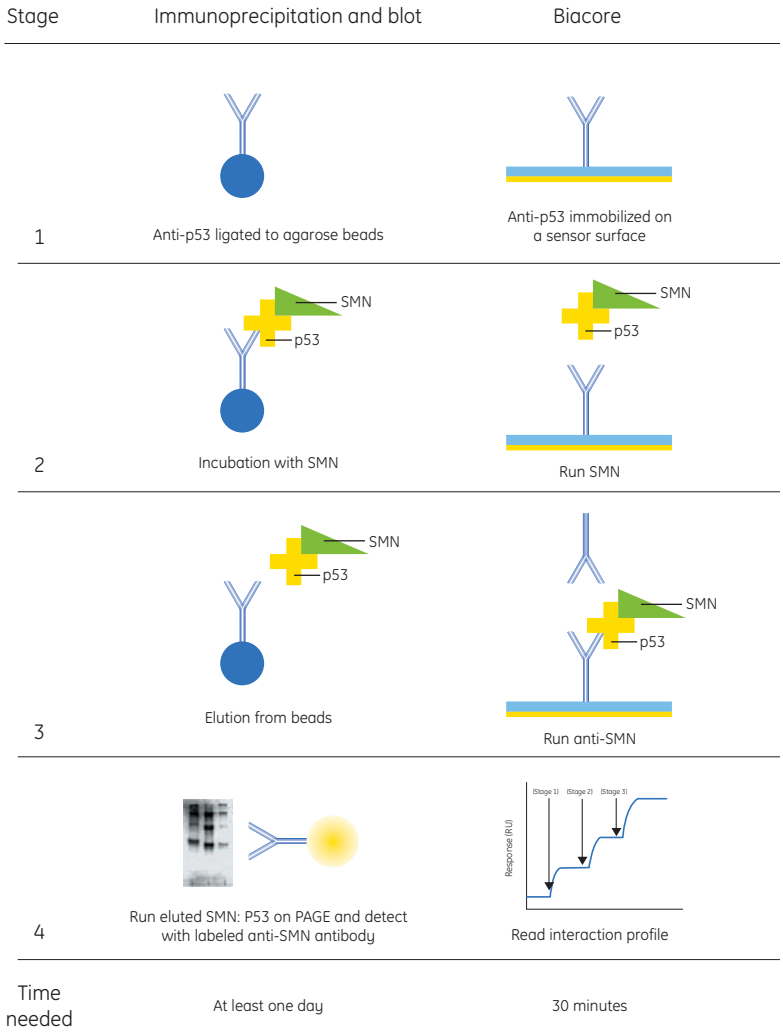


Fig 7. Comparison between immunoprecipitation/blotting and a Biacore system-based procedure in the detection of molecular complexes. Both assays reveal that SMN protein derived from whole cell extracts is associated with p53 in the cell. Results achieved by immunoprecipitation are available within one day whereas a Biacore assay takes minutes.

Molecular consequences of disease-causing mutations

Mutant SMN proteins from patients were tested for p53 binding. Two SMN proteins with mutations found in patients with severe type I SMA and a peptide from a patient with less severe type II SMA were immobilized on a sensor surface and exposed to p53. The extent of reduction in SMN peptide binding strongly correlated with the clinical subset. This defect is probably attributable to the inability of the SMN mutant proteins to efficiently self-associate. Young *et al*. then applied the sequential Biacore assay approach to show that it is this very inability to self-associate that may prevent SMN from sequestering and inhibiting p53 function. In these experiments, p53 failed to bind to immobilized monomeric SMN and was only induced to bind when additional dimeric SMN was immobilized on the same sensor surface (Table 2 and Fig 8).

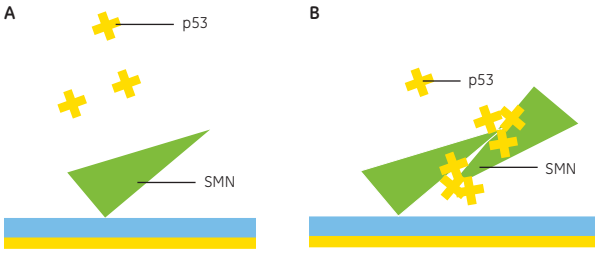


Fig 8. Non-self associated SMN cannot sequester p53. A Biacore assay was used to show that p53 bound only weakly to monomeric SMN (A), a configuration that is likely to dominate in SMA patients of various degrees of severity (see Table 2). When dimeric SMN was immobilized on a sensor surface (B), p53 binding was greatly increased.

Table 2. Monitoring self association of SMN protein on a sensor surface

SMN protein form	Binding to wildtype SMN (% of wildtype)
Wildtype	100%
Truncated SMN[1]	33%
Two type I mutations[2]	20% and 31%
Type II mutation[3]	57%

[1] Truncated SMN is the primary product of the SMN2 allele, which is present in SMA patients.

[2] Type I mutations were isolated from severe SMA.

[3] Type II mutations were isolated from mild SMA.

These findings are supported by results from indirect confocal immunofluorescence microscopy experiments showing that SMN and p53 co-localize in Cajal bodies (sites of small nuclear ribonucleoprotein particles involved in RNA splicing) of normal fibroblasts, but not in those derived from SMA patients. In the patient fibroblasts, SMN levels are greatly reduced and in this cellular context, activated p53 can be observed within the nucleolus, suggesting that SMN may play a role in targeting p53 to Cajal bodies.

Selecting antibodies as molecular probes

To determine a possible mechanism to explain why mis-sense mutations in SMN should compromise protein function and hence cause disease, label-free protein interaction analyisis was used to discover how native peptide fragments of SMN interact with each other (10). 22 monoclonal antibodies were raised to full-length SMN protein and tested for immunoreactivity against the peptides encoded by each exon in the cloned SMN gene using a Biacore system. The antibodies were immobilized on a sensor surface and screened with all fragments. Antibodies were thus identified that specifically recognized certain peptides within the protein and that could then be used to characterize intra-chain interactions that may determine conformational settings of functional importance in the full-length protein. By immobilizing each peptide fragment and screening with exon-defined peptides, the authors were able to identify fragments of SMN that possessed the potential to interact with each other.

Figure 9 shows the construction of an experiment that provided a wealth of information about the molecular events underlying the function of this protein. A monoclonal antibody that recognized the peptide product of exon "A" alone was immobilized on sensor surface. Subsequent injections of different peptides showed that the capture of "A" enabled peptides "B1" and "B2" to bind sequentially in the same experiment. These data show not only that "A" appears to form a scaffold to allow the assembly of B1 and B2, but that the functional protein product is a result of the creation of a novel epitope formed by the combination of B1 and B2.

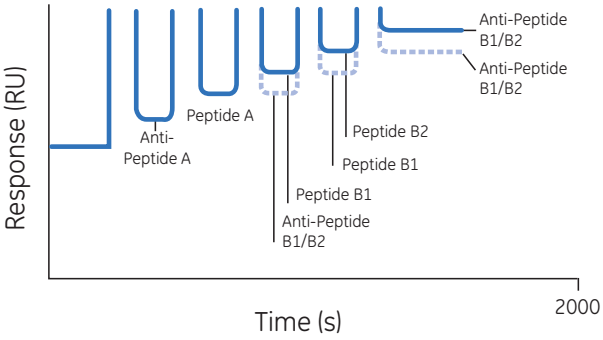


Fig 9. Interaction profile showing how, by using a combination of carefully specified monoclonal antibodies and recombinant peptide fragments, a Biacore assay can provide a powerful means of helping to determine the order of assembly of protein structures.

References

9. Young, P. J. *et al.*
   A direct interaction between the survival motor neuron protein and p53 and its relationship to spinal muscular atrophy.
   J. Biol. Chem. **277**, 2852–2859 (2002).

10. Young, P. J. *et al.*
    The exon 2b region of the spinal muscular atrophy protein, SMN, is involved in self-association and SIP1 binding.
    Hum. Mol. Genet. **9**, 2869–2877 (2000).

The development of humanized antibodies for neuroblastoma therapy

Steering antigen orientation in assays for antibody characterization

- Characterize antibodies with confidence by presenting antigens at a defined density and orientation on a sensor surface
- Rapidly uncover unexpected changes in interaction profiles due to small engineered changes in molecular structure

Gangliosides are membrane-spanning glycolipids and are enriched in neurons. Their expression is associated with certain neuropathies including human neuroectodermal cancers and Alzheimer's disease. A Biacore system was used to develop a simple assay for the detection of anti-ganglioside antibodies (11). One requirement of the technique is that the gangliosides are presented in a consistent and appropriate orientation for antibody characterization. To achieve this, the gangliosides were immobilized via the lipid tail. The authors present a simple and rapid protocol in which a stable sensor surface is created that can be readily reconditioned and reused.

Phase I clinical trials, in which humanized anti-ganglioside monoclonal antibodies were tested in children with neuroblastomas, have shown that a host immune response is induced against the murine component of the antibody. For this reason, it is desirable to engineer fully humanized antibodies that retain the murine specificity-determining regions, but from which the flanking V- region sequences have been replaced with human counterparts (12). Nakamura *et al*. based the characterization of the interaction between these humanized antibodies and immobilized gangliosides GD2 and GD3 on the protocol of Catimel *et al* (11). Label-free interaction analysis revealed that humanization altered the association and dissociation rates of the antibodies in ways that are difficult to predict and provided information about the molecular mechanisms underlying functional changes in humanized antibodies.

References

11. Catimel, B. *et al.*
Direct immobilization of gangliosides onto gold-carboxymethyldextran sensor surfaces by hydrophobic interaction: applications to antibody characterization.
Glycobiology **8**, 927–938 (1998).

12. Nakamura, K. *et al.*
Construction of humanized anti-ganglioside monoclonal antibodies with potent immune effector functions.
Cancer Immunol. Immunother. **50**, 275–284 (2001).

Neurotransmitter release

Using interaction data from label-free interaction analysis to explain function in cell-based assays

- Study protein interactions involved in synaptic vesicle: plasma membrane fusion in neurons on a sensor surface

Concise affinity data measurements on Biacore systems can be given functional significance in cell-based assays. Quetglas *et al*. used a Biacore system to provide quantitative affinity data in support of a highly credible hypothesis for the regulation of neurotransmitter release (13).

A Biacore system was used to reveal the molecular mechanisms driving the fusion of neuronal synaptic vesicles with the plasma membrane to release neurotransmitters. Vesicle-associated membrane protein (VAMP-2 or synaptobrevin) is anchored in the vesicle membrane and can associate with two proteins in the plasma membrane, SNAP-25 and syntaxin-1. In the presence of Ca^{2+}, the two membranes are thus brought together to promote fusion of the lipid interfaces. Calmodulin, a cytosolic calcium sensor protein, is implicated in several membrane fusion processes in other mammalian models and in yeast. Specifically, Stephanie Quetglas and colleagues (INSERM Unit 464) in collaboration with Raymond Miquelis (UMIM) at the IFR Jean Roche, Université de la Méditerranée in Marseille have shown that calmodulin binds to a C-terminal VAMP-2 sequence located close to its anchor in the vesicle membrane. They used cell-based assays and Biacore assays to test some of the discrete molecular steps within this process. Their work with protein: protein interactions as well as protein: lipid interactions, indicates the broad scope of label-free protein interaction analysis.

The role of Ca^{2+} in interactions between calmodulin and VAMP

The requirement for Ca^{2+} to support calmodulin binding to VAMP was tested by injecting calmodulin over a VAMP captured on a sensor surface in the presence of increasing concentrations of Ca^{2+}. Significant binding occurred only in the presence of Ca^{2+} at concentrations above 0.3 µM. Mutations, designed according to consensus sequences in other calmodulin-binding proteins, were tested for their capacity to inhibit calmodulin binding. Compared with the wildtype peptide, those bearing mutations aimed at eliminating basic charges or in altering the hydrophobic quality of the consensus were severely impaired in their affinity for immobilized calmodulin (10-fold and 300-fold inhibition, respectively). In a further Biacore assay, liposomes were immobilized on a sensor surface and shown to bind VAMP-derived peptides. Again the mutants, particularly those with reduced hydrophobic quality, bound lipids only weakly. Finally, in a competition assay in which VAMP was linked to agarose beads, increasing concentrations of calmodulin were able to displace labeled liposomes bound to VAMP in the presence of Ca^{2+}. Taken together, these results obtained using label-free protein interaction analysis suggest that lipids and calmodulin share a binding motif on VAMP.

Vesicle fusion and exocytosis

The mutants were then tested for function in two cell-based assays. Firstly, neuroendocrine cells were pulsed with 100 mM KCl and release of the neurotransmitter, catecholamine, was measured. Catecholamine release was reduced by 80% in the presence of wildtype calmodulin-binding peptides, suggesting that the VAMP peptides sequestered endogenous calmodulin and prevented exocytosis. In contrast, mutant VAMP peptides (non-calmodulin-binding) affected exocytosis only very weakly.

Secondly, PC12 cells were treated with tetanus toxin, which cleaves VAMP at a site immediately N-terminal to the calmodulin binding site, inhibiting vesicle fusion and hence exocytosis. When cells were transfected with mutant VAMP that cannot be cleaved by the toxin but which still binds calmodulin, membrane fusion and exocytosis were restored. However, the introduction of additional mutations (i.e. those which inhibited calmodulin and lipid binding in Biacore assays) abolished rescue of Ca^{2+}-dependent exocytosis.

Reference

13. Quetglas, S. *et al.*
Calmodulin and lipid binding to synaptobrevin regulates calcium-dependent exocytosis.
EMBO J. **21**, 3970–3979 (2002).

Receptor: ligand interactions and signal transduction

Translating kinetic data from label-free protein interaction analysis to an understanding of the regulation of neural networks

- Use kinetic information to provide clues on how neurons cope with processing extremely large information loads
- Mimic the acidic environment of endosomes or the conditions of cerebral acidosis by varying the pH of the running buffer

Although the molecular information carriers of signal transduction pathways are highly conserved across cell types and operate by similar mechanisms such as tyrosine phosphorylation and dephosphorylation, it is perhaps the transmission of information between and within the neurons of mammalian nervous systems that the performance of these pathways is pushed to almost unimaginable levels of traffic volume while at the same time demanding exquisite regulation. The following reports illustrate how precise kinetic data from label-free protein interaction analysis are helping to lay the foundation of knowledge that will reveal the molecular basis for the regulation of complex neural networks.

Kinetic analysis of the interaction between Shh and megalin

The pleiotropic signaling molecule, sonic hedgehog (Shh) is involved in several aspects of neurodevelopment including maturation of ventral neural cells and patterning of the ventral neural tube. It functions by binding to a receptor called Ptc, which is normally embedded in the neural cell membrane in close association with a signal transducing co-receptor called Smo, an interaction that maintains Smo in a dormant state. On binding to Shh, however, the membrane-bound complex dissociates and leaves Smo in a monomeric and active state.

Megalin, a member of the LDL receptor family is critical in neurodevelopment. The phenotype of megalin deficiency is similar to mice deficient in Shh or Smo, including apoptosis of neural epithelial cells. Scott Argraves and colleagues at the Medical University of South Carolina in Charleston showed that megalin was co-expressed during neural development with Shh or known receptors for Shh and thus suggested that a functional relationship may exist between Shh and megalin (14).

The Argraves group performed a kinetic analysis of the interaction between the biologically active N-terminus of Shh and megalin using a Biacore system. Megalin was immobilized on a sensor surface and a titration series of Shh was passed over the prepared surface. The effect of pH on the dissociation rate of the complex was tested by washing in buffers of varying acidity. Acidic pH made little difference to the dissociation rate suggesting that the complex would remain stable within the acidic environment of endosomes. It is therefore possible that the Shh: megalin complex functions in endosome-mediated transcytosis to enable distal signaling. This might possibly be a mechanism for cell differentiation in development e.g. in the maturation of cells in the ventral region of the neural tube that differentiate to ventral nerves, a process dependent on both Shh-mediated signal transduction and megalin expression.

Purkinje cell function

The glutamate receptor δ2 (GluRδ2) is exclusively expressed on the dendritic synapses of Purkinje cells, structurally unique cells that are among the most organizationally complex neurons in the mammalian nervous system. Although they receive an enormous amount of data from neurons of the spinal cord and elsewhere, they are nevertheless able to convert this input into interpretable information and are, in fact, the sole channel of output from the cerebellar cortex. That GluRδ2 is limited to these cells possibly implies a function in this extraordinary feat of information processing. Susumu Kawamoto and his research group at Yokohama City University School of Medicine showed that an intracellular protein called delphilin co-localizes with GluRδ2 at parallel fiber (input)-Purkinje cell (output) synapses (15). Analysis using label-free protein interaction analysis showed that recombinant delphilin PDZ domains bind to the C-terminus of GluRδ2 with moderate affinity. They also showed that delphilin bound profilin, an actin-binding protein in an interaction with a very slow dissociation rate. It is possible, therefore, that delphilin acts as a molecular bridge linking GluRδ2 to the actin cytoskeleton via profilin with rapid turnover of the GluRδ2: delphilin interaction contributing to the capacity of the Purkinje cell to process a very large amount of input data.

Physiological functions of nerve growth factor

The physico-chemical composition of the microenvironment of the binding site between ligand and receptor, or "chemical space", has significant effects on protein interactions. Ionic strength and pH, for example, affect non-covalent bonds and so by varying buffer composition, judgments can be made on the nature of the interaction.

Label-free interaction analysis was used to find out if local variations in pH changed the capacity of Zn^{2+} and Cu^{2+} to modulate nerve growth factor (NGF) binding to its receptor, TrkA (16). To do this, Zn^{2+} and Cu^{2+} were included in running buffers with pH varying from 5.5 to 7.4, mimicking local conditions in cerebral acidosis, a condition frequently arising after stroke or traumatic insult. This pH range also covered the pKa value of histidine, the position and electrostatic status of which are known to influence cation binding.

The authors prepared and immobilized recombinant TrkA on a sensor surface. The binding affinity for the interaction between NGF and TrkA was calculated over a pH range covering the pKa value of histidine. This revealed that Zn^{2+}, but not Cu^{2+}, lost its ability to bind NGF and inhibit its interaction with TrkA under acidic conditions. The physiological consequences of this may depend on the cell type and context; TrkA-expressing cells in an acidic environment and needing NGF for survival may well benefit from the inactivation of Zn^{2+}, whereas the effects might be detrimental if NGF, in contrast, initiates signals leading to cell death.

References

14. McCarthy, R. A. *et al.*
Megalin functions as an endocytic sonic hedgehog receptor.
J. Biol. Chem. **277**, 25660–25667 (2002).
15. Miyagi, Y. *et al.*
Delphilin: a novel PDZ and formin homology domain-containing protein that synaptically colocalizes and interacts with glutamate receptor delta 2 subunit.
J. Neurosci. **22**, 803–814 (2002).
16. Ross, G. M. *et al.*
The binding of zinc and copper ions to nerve growth factor is differentially affected by pH: implications for cerebral acidosis.
J. Neurochem. **78**, 515–523 (2001).

GE Healthcare Bio-Sciences AB
Rapsgatan 23
751 84 Uppsala
Sweden

www.biacore.com

Previously published March 2004

imagination at work

28-9383-25 AA 05/2008