Cancer research and proteomics

Label-free interaction analysis: revealing the secrets of biomolecular interactions
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A database of research papers describing applications using label-free interaction analysis is continually updated and presented on www.biacore.com.
Apoptosis

Apoptosis, or programmed cell death, is a normal process in tissue homeostasis. The avoidance of apoptosis is characteristic of tumor cells, enabling them to survive during metastasis, the period during which the tumor cell migrates from its origin and, as the cell enters the circulation, where it is temporarily free from attachment to a matrix.

Several groups have used Biacore systems to investigate the molecular interactions that comprise apoptotic signaling pathways and the mechanisms that enable a transformed cell to escape apoptosis. This work has lead to the endorsement of promising strategies in the search for new pro-apoptotic molecular targets.

Using kinetics to optimize efficacy of therapeutic apoptotic antibodies

- Binding kinetics of a fully human bispecific fusion protein to parent antigens demonstrates that the fusion process does not significantly alter the interaction profile of the component proteins

A fully human gene-fused bispecific antibody was developed from two single chain Fv (scFv) molecules. One human anti-CD16 scFv component, NM3E2, was isolated from a human scFv phage display library, while a second was used as a fusion partner. A Biacore system was used to measure the binding kinetics of the bispecific fusion protein to either of the parent antigens (1). The experiments showed that the fusion process did not greatly alter the interaction profile of the component scFvs. A Biacore "sandwich" assay demonstrated that the fusion protein, immobilized on a sensor surface via HER2/neu, also bound CD16 (Fig 1 and 2).

In an in vitro assay, the presence of the fusion protein increased the efficiency with which peripheral blood lymphocytes induced lysis of human ovarian cancer cells over-expressing HER2/neu.

This fusion protein, due to its human origin, may overcome some of the potential immunogenic problems associated with biotherapeutics derived from murine proteins. The fusion protein is easy to produce, demonstrates a high level of in vitro tumor cell cytotoxicity and is highly selective for tumors.

Reference

Inhibiting tumor cell growth and increasing sensitivity to radiotherapy and chemotherapy with anti-cancer peptide mimetics

- Slow dissociation rate of the interaction between a peptide mimetic and recombinant tumor marker indicates the formation of a stable complex
- Efficient induction of tumor cell apoptosis indicates that the mimetic may be effective in the treatment of breast, ovarian and colon cancer

Human breast, ovarian and colon cancer cells overexpress an oncogenic variant of HER2/neu, a member of the family of growth factor receptors that includes the EGF receptor. Monoclonal antibodies to the ectodomain of HER2 have been shown to reverse the malignant phenotype. However, the use of murine antibodies is limited due to immunogenic responses by the host. In addition, the large size of such reagents limits their feasibility as biotherapeutics.

In order to circumvent these limitations, Park et al. designed a short peptide mimetic of 1.5 kDa, based on a structural analysis of the complex formed between anti-HER2 and HER2 [2]. Like its immunoglobulin counterpart, the mimetic induced tumor cell apoptosis, inhibited tumor cell proliferation, colony formation and the growth of solid tumors in vivo.

A Biacore system was used to define the kinetic profile of the interaction between the peptide mimetic and recombinant HER2 extracellular domain immobilized on a sensor surface. Although the affinity of the mimetic for the tumor marker was somewhat lower than that of the antibody, the dissociation rate was similar, indicating a stable complex and hence a promising therapeutic alternative.

Reference
2. Park, B. W. et al.

Charting the signaling pathways leading to apoptosis

- Signaling pathways are revealed by studying interactions of point mutated apoptotic proteins with cell surface receptors

The apoptotic protein, LIGHT, is known to bind two cellular receptors, lymphotxin-β receptor (LTβR) and the herpes virus entry mediator (HveA). By introducing point mutations in peptide loops thought to be involved in receptor binding, changes in LIGHT binding characteristics were achieved in a Biacore-based interaction study [3].

The interaction of native and mutant LIGHT to both of these receptors was studied. HveA and LTβR were immobilized on a sensor surface using an antibody capture technique. One mutant was shown to bind HveA but not LTβR and did not lead to apoptosis of target cells when tested in an in vitro assay. These results strongly suggest that at least two distinct signaling pathways are activated via the interaction of LIGHT with its cell surface receptors, and indicate that only one pathway leads to apoptosis.

Reference
**Signal transduction**

Defining the multiple signaling pathways that lead from the extracellular environment to control cell cycle, gene transcription, cell division and apoptosis will guide cancer researchers to design antagonists that pinpoint and inhibit key molecular interactions in these cascades.

Label-free interaction analysis using Biacore systems is increasing our scope to interpret function from the structure of specific molecules involved in signaling. The hope is that new therapeutic opportunities will follow and Biacore systems have made a major contribution to each of the studies summarized below.

**Using liposomes in a Biacore-based interaction assay to show how a lipid microenvironment is vital for the functional integrity of a signaling protein**

- Proteins requiring a membrane-like environment for their function can be studied in a Biacore assay using a lipid bilayer or liposomes immobilized on a sensor surface
- Anti-cancer biotherapeutics may be developed by inhibiting the integration of Ras protein into cell plasma membranes

Ras is a GTP-binding protein involved in signal transduction. Signaling pathways initiated by receptor tyrosine kinases via Ras are transduced across the nuclear membrane by activating the MAP-kinase pathway. This cascade of threonine kinases includes Raf, the first enzyme in the series to which activated Ras can bind. Ras is active when bound to GTP and inactive when GTP is hydrolyzed to GDP. Oncogenic mutations in the Ras gene can induce cell transformation or differentiation and some cell lines rapidly differentiate on microinjection of oncogenic Ras.

In addition to being in a GTP-bound state, Ras must be immobilized in the plasma membrane to bind Raf. Drugs that impair or inhibit the stability of Ras in plasma membranes are therefore of interest. In a study by Bader et al., a synthetic lipid was linked to Ras and allowed to interact with a lipid bilayer on a sensor surface (4).

As the synthetic lipid was made more hydrophobic, the stability of the insertion increased. Injected Raf only bound the lipid-anchored Ras associated with GTP (Fig 3).

Functional confirmation of this...
interaction was shown when microinjection of truncated oncogenic Ras (which is unable to bind to the plasma membrane) failed to induce rat pheochromocytoma cells to differentiate; the cells only transformed if the truncated Ras was co-injected with a lipid anchor.

**Reference**

**Establishing the mechanisms supporting the functions of proteins involved in cell division**

- Interaction data obtained by yeast two hybrid analysis and co-immunoprecipitation are confirmed and quantified using Biacore systems
- Analysis of splice variants in Biacore assays reveals protein domains essential for binding and function

Adenomatous polyposis coli (APC) is a tumor suppressor gene involved in familial and sporadic forms of colorectal cancer. The function of APC is unknown although it interacts with other intracellular proteins including tubulin. Erdmann et al. propose that APC may form a scaffold for the assembly of a number of signaling molecules and may also regulate the phosphorylation status of these interacting proteins.

This paper describes the interaction between APC and the protein tyrosine phosphatase, PTP-BL. Label-free interaction analysis using a Biacore system was used to quantify the binding of APC to PDZ domains of PTP-BL. APC was immobilized on a sensor surface and two alternative PDZ domain splice variants, PDZ2a and PDZ2b, were injected over the immobilized peptide (5). PDZ2a, but not PDZ2b, was shown to bind APC (Fig 4). The kinetics of the PDZ2a:APC interaction were investigated and were shown to be close to the values of other PDZ interactions reported in the literature.

This work shows that APC and PTP-BL interact and that APC probably modulates tyrosine phosphorylation on interacting proteins. These findings, taken together with the other known targets of PTP-BL, may help guide the development of biotherapeutics aimed at modulating many cancer-related cellular functions like migration, adhesion and cell cycle control.

![Fig 4. PDZ2a (1), but not PDZ2b (2), specifically binds APC.](image)

**Reference**
Translating kinetic information to function in signal transduction

- Kinetics of specific protein interactions in signaling pathways directly impact on the regulation of mitogenesis.

Epidermal growth factor (EGF) induces its biological effect through interaction with the EGF receptor, a member of the erbB family of receptors. The erbB oncogene encodes a truncated form of the EGF receptor with a continuously active intracellular tyrosine kinase domain; cells expressing this oncogene behave as if constantly being signaled to proliferate.

In a study by Lenferink et al., chimeras comprising EGF and TGF\(\alpha\), both of which bind erbB, were constructed (6). The chimeras possessed at least tenfold more mitogenic activity than either of the wild types. The extracellular domain of EGF receptor was immobilized on a sensor surface and chimeras or wildtype controls were injected over the surface (Fig 5). Interactions involving all three chimeras were characterized by faster on and off rates when compared with either of the wildtype controls.

The enhanced ability of the chimeras to induce MAP kinase phosphorylation was strongly associated with faster association with the receptor. The authors speculate that more intense receptor signaling is induced by ligands with rapid association and dissociation kinetics.

![Diagram](image)

**Fig 5.** EGF/TGF\(\alpha\) chimeras bind EGFR with similar affinity to wild type controls but with faster on and off rates.

**Reference**

Superagonistic activation of ErbB-1 by EGF-related growth factors with enhanced association and dissociation rate constants.
Ligand fishing and biomarker screening

Searches of genomics databases are leading to the discovery of potential new cell surface receptors. Understanding their function, however, is limited until the ligand that binds to them is discovered. Ligand fishing is the process by which 'orphan' receptors are screened against a multitude of compounds or cell extracts in order to reveal possible ligands.

**Identifying putative ligands for cell surface receptors from cell-conditioned media**

- Ligand fishing on Biacore systems is a first step prior to large scale purification for biochemical and functional characterization

A fusion protein containing the extracellular domain of the cell surface receptor, Flt4 was immobilized on a sensor surface (7). Concentrated media from over 100 different cell lines were injected over the surface (Fig 6). When the media were tested in a biological assay, only those that bound Flt4 in Biacore assays induced tyrosine phosphorylation of Flt4 in CHO cells stably transfected with receptor.

Three peptides that correlated with Flt4-binding activity were then selected after SDS-PAGE of fractions eluted from affinity columns.

When degenerate PCR primers based on partial amino acid sequences of two of the fractions were constructed, a 65 bp product was amplified and sequenced and was shown to contain a precise match to the known amino acid sequence. This cDNA was used to characterize the ligand for Flt4, verify the function of the protein in newly transfected cells, and identify areas of homology on other functionally related growth factors and to study ligand gene expression.

![Fig 6. Three cell-conditioned media (CM) specifically bind Flt4-Fc. When CM is co-injected with excess Flt4-Fc (but not human IgG or KIT-Fc), the signal is inhibited.](image)

**Reference**

**Screening clinical samples for the presence of biomarkers**

- Biacore assay reveals false positive and negative results in a similar test performed using ELISA.

Recurrent arterial or venous thrombosis is associated with heterozygous congenital protein S (PS) deficiency. Thrombosis occurs in approximately 20 to 30% of systemic lupus erythematosus (SLE) patients and is closely associated with the presence of anti-PS autoantibodies.

In a study by Guermazi et al., purified PS was immobilized on a sensor surface (8). Plasma from SLE patients was then injected over the surface, followed by anti-human IgG to test for the presence of anti-PS antibodies (Fig 7). Note that Biacore assays revealed false positive and false negative results (Fig 8) when the same samples were tested using an ELISA. Six of 27 samples tested were found to be positive for anti-PS.

The application shows how the principle of ligand fishing was extended to a clinical application where a purified target, in this case a defined autoantigen, is used as bait to retrieve and identify autoreactive antibodies from a complex mixture of proteins in human serum.

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**Fig 7.** The presence of anti-PS antibodies from patients’ sera bound to immobilized PS is detected using Fc-specific goat anti-human IgG.

**Fig 8.** Real time profiles of interactions between anti-PS and PS. That the signals are caused by interactions specifically involving anti-PS and PS is confirmed by subsequent injection of Fc-specific anti-human IgG (arrow).  
Profile 1 = negative by Biacore assay  
Profile 2 = positive by Biacore assay  
Profile 3 = positive by Biacore assay (false negative by ELISA)  
Profile 4 = negative by Biacore assay (false positive by ELISA)

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**Reference**

Further evidence for the presence of anti-protein S autoantibodies in patients with systemic lupus erythematosus.  
Protein: protein interactions

Defining the stability of interactions and mapping the organization of proteins within a complex, together with the identification of specific amino acid residues involved in these interactions, is fundamental to comprehending structure-function relationships. Label-free interaction analysis using Biacore systems provides data for protein interactions, from studies of binding sites to interactions within multi-protein complexes.

Defining the functions of interacting proteins in multimolecular complexes through interaction profiling

- Discover how multi-protein complexes are constructed
- Interaction profiles help interpret the functions of proteins within a complex

The synthesis of aminoacyl tRNA is catalyzed by an enzyme complex consisting of three core proteins and additional peripheral proteins imparting amino acid specificity. In a study by Robinson et al., a histidine-tagged form of one core protein, p38, was immobilized on a sensor surface (9). Peripheral proteins were prepared at different concentrations and injected over the prepared surface (Fig 9). The peripheral protein, lysyl-RS, formed a stable complex with p38 under buffer conditions in which lysyl-RS was dimeric. Under buffer conditions in which lysyl-RS was monomeric, however, the affinity was greatly reduced, principally due to an increased rate of dissociation.

In contrast to lysyl-RS, the peripheral protein, aspartyl-RS bound sequentially to p38 as two monomers. The interaction with p38 could be described by simple kinetics (dimeric aspartyl-RS to dimeric p38) only if ATP and Mg^{2+} were added to the running buffer, which supported the formation of a stable dimer.

The peripheral protein, glycyl-RS bound p38 very weakly but when p38 was first reacted with p43 and arginyl-RS, the complex was stable, indicating the order of construction of the complex. Lateral interactions may thus contribute to the stability of the complex (Fig 9).

No single peripheral protein was able to competitively displace another, indicating discrete binding sites on p38 for each of its partners. The authors used the data obtained from Biacore experiments as a blueprint to successfully “rebuild” an enzyme complex in vitro.

Fig 9. Lateral interactions in protein complex; the stability of the interaction between p38 and glycyl-RS is increased if p38 firstly binds p43 and arginyl-RS.

Reference

Using label-free interaction analysis and a random mutant library to deconstruct binding sites on proteins

- “Functional mutants” are screened using antibodies immobilized on a sensor surface to identify single dominant amino acids in a binding site
- Simple epitope mapping studies are performed by sequential injection of antibody 1-antigen-antibody 2 on a sensor surface

The identification of specific amino acid residues involved in protein interactions is important for understanding the relationship between molecular structure and function. A Biacore system was used to investigate the binding of 27 randomly selected mutant plasminogen activator inhibitor-1 (PAI-1) phage display clones to four different classes of monoclonal anti-PAI-1 immobilized on a sensor surface (10).

As all the mutants were impaired in their binding to as many as three antibodies, it was sufficient to select a relatively few number of clones to find functional binding mutants. The amino acid sequences of the non-binding clones were aligned and certain common amino acid mutations were found that were entirely missing or rare in binding counterparts.

The functional impairment of the phage display mutations was tested by the construction and expression of whole PAI-1 protein containing these point mutations. When the whole protein mutants were analyzed for binding to the antibodies, a similar impairment in binding to the antibodies was seen.

A 3D map of PAI-1 showed that all the identified epitopes were located in different regions of the protein. In an epitope mapping study, monoclonal anti-PAI-1 was immobilized on a sensor surface and wildtype PAI-1 was captured. When two further antibodies were passed over the complex, both bound PAI-1, indicating that no binding sites overlapped (Fig 10 and 11).

**Reference**

Discovering how multicomponent protein complexes regulate biological processes

- Analysis of multiple reciprocal interactions provides a basis for understanding how the alternative complement pathway distinguishes between foreign and self-tissues

The alternative complement protein, C3b, is partly regulated by interactions with the plasma protein, factor H (fH). The interaction sites between fH and surface-bound C3b were characterized in efforts to understand how the alternative complement pathway is activated only by foreign tissues (11).

C3b, along with its proteolytic fragments C3c and C3d were immobilized on a sensor surface. Eight fH constructs were injected over the immobilized components and three distinct binding sites for C3b on fH were mapped.

Each fH construct interacted with a distinct site on C3b. Site 1 bound to intact C3b only, site 2 bound to C3b and C3c and site 3 bound to the C3b and C3d fragments (Fig 12). Multiple reciprocal interactions between C3b and fH may provide a basis for the exclusive reactivity of the alternative pathway with foreign and host structures.

Fig 12. fH site 3 binds to intact C3b and its sub fragment C3d only.

Reference
   Each of the three binding sites on complement factor H interacts with a distinct site on C3b.
Biacore systems and mass spectrometry

Molecules captured by a ligand bound to a sensor surface may be proteolytically digested and the resulting mixture of peptides recovered and analyzed using ESI-MS/MS (tandem electrospray ion trap MS). Biacore systems combined with MS/MS form a versatile system for the discovery and identification of novel biomolecular interactions.

**Combining label-free interaction analysis with mass spectrometry**

- Captured proteins are isolated and sequenced in a single analysis

A novel analysis procedure was developed to combine a Biacore system with ESI-MS/MS for the isolation and sequencing of captured proteins (12). The technique provides a means of detecting, capturing and delivering mid-to-low femtomole amounts of protein.

In one of two examples from this paper, inositol triphosphate (IP3) was immobilized on a sensor surface and lysates from bacteria transfected with IP3-binding protein were injected over the sensor surface. An air partition method was then used to deliver minute volumes of enzyme to digest the bound protein. The eluted peptides were then collected directly in a reverse phase capillary column to remove contaminants that may interfere with the MS analysis (Fig 13). The eluted peptides were analyzed using an HPLC-ESI-MS/MS system, which confirmed the presence of IP3 binding protein.

**Fig 13.** The principle of on-surface digestion. Protein bound to the sensor surface is enzymatically digested in situ and captured in a capillary column before MS/MS analysis.

**Reference**
