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# Detection of bound and free IGF-1 and IGF-2 in human plasma via biomolecular interaction analysis mass spectrometry

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**Abstract Insulin like growth factor (IGF)-1 and IGF-2 were assayed from human plasma via biomolecular interaction analysis mass spectrometry, utilizing antibodies as ligands for affinity retrieval. Detection of both targeted and non-targeted IGFs in the mass spectra indicated possible protein complex retrieval by the individual antibodies. A series of control experiments eliminated the possibility of analyte cross-walking between flow cells, significant antibodies cross-reactivity, and direct IGF interactions. To disrupt the putative protein complex and release its constituent proteins, plasma samples were treated with detergents. An SDS-treated plasma yielded IGF signals in a different ratio than the one observed in the mass spectra from the non-treated plasma, suggesting disruption of the protein complex, and its retrieval from non-treated plasma. Novel truncated IGF-2 variant, missing its N-terminal Alanine, was detected in all mass spectra.**


*Key words:* Biomolecular interaction analysis mass spectrometry; Surface plasmon resonance; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; IGF-1; IGF-2; Protein complex

## 1. Introduction

The insulin like growth factors 1 and 2 (IGF-1 and IGF-2) are members of an important network of proteins that regulate metabolic, growth, and other cellular processes and activities [1]. Primarily synthesized in the liver, the IGFs circulate in serum in a form of protein complexes, bound to IGF-binding proteins (IGFBPs). Less than 1% of the IGFs circulate in free, unassociated form. The binding to the IGFBPs increases the half-life of IGFs in blood, whereas the physiological role of the free IGF has not yet been determined. Structurally, IGF-1 and IGF-2 share 62% amino acid sequence homology, and there is 40% homology between the IGFs and proinsulin.

Immunoassays (ELISA, radio, or chemiluminescence) are generally used for assaying IGFs in plasma/serum. Because the concentration of free IGFs in serum samples can increase upon storage (due to proteases-induced release of the bound IGFs), determination of the total IGF is preferred in clinical research and practice. Acid ethanol extraction is commonly used to release the bound IGFs prior to assaying, although additional steps are often required to minimize the IGFBPs interference [2,3]. IGFs measurements are routinely performed using commercially available immunoassays [4], and recently studies on large populations have yielded important correlations between increased IGF concentrations and the risk of cancer [5–7].

In this work we explore the detection of both free and bound IGFs via biomolecular interaction analysis mass spectrometry (BIA/MS) [8–13]. In its core, BIA/MS is a synergy of two individual technologies: surface plasmon resonance (SPR) sensing [14,15] and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [16,17]. Each technology brings a unique dimension to the BIA/MS analysis: SPR is employed for protein quantification, whereas MALDI-TOF MS is utilized to delineate structural features of the analyzed biomolecules. Due to the non-destructive nature of the SPR detection, in-vivo assembled protein complexes can be analyzed in the SPR Biosensor and subsequently delineated in the MALDI-TOF MS analysis [18]. In this work we assayed the IGFs utilizing antibodies as ligands for affinity retrieval of the proteins in their complexed and free forms from human plasma.

## 2. Materials and methods

### 2.1. Proteins and antibodies

Affinity-purified rabbit anti-human IGF-1 antibody (anti-IGF-1) was obtained from Cell Sciences (Norwood, MA, USA). Mouse anti-human IGF-2 antibody (anti-IGF-2), and recombinant IGF-1 and IGF-2 were purchased from US Biological (Swampscott, MA, USA).

### 2.2. Samples

Human blood was obtained from a single subject recruited within Intrinsic Bioprobes Inc. (IBI), following a procedure approved by the IBI's Institutional Review Board, and after signing of an Informed Consent form. In short, 45 μl human blood was drawn under sterile conditions from a lancet-punctured finger with heparinized microcolumn (Drummond Scientific Co., Broomall, PA, USA), mixed with 200 μl of HEPES buffered saline (HBS-EP) buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% (v/v) polysorbate 20, 3 mM EDTA) and centrifuged for 30 s (at 7000 rpm, 2500×*g*) to pellet the red blood cells. The supernatant (plasma) was used as is, or it was diluted five-fold with HBS-EP buffer (for a final dilution of 50×)

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*Abbreviations:* BIA/MS, biomolecular interaction analysis mass spectrometry; FC, flow cell; HBS, HEPES buffered saline; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; RU, response unit; SPR, surface plasmon resonance

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### 2.3. SPR analysis

A Biacore X instrument (Biacore AB, Uppsala, Sweden) was utilized for the first dimension of BIA/MS (affinity retrieval and SPR quantification). CM5 Research Grade Sensor Chips (carboxymethyldextran derivatized surface, Biacore AB) were used in the experiments, with HBS-EP running buffer at a flow rate of 5 μl/min. The proteins were immobilized on sections (flow cells, FC) on the chip surface following a standard EDC/NHS (*N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide) coupling protocol [19]. Regeneration of the protein surfaces was achieved via short injections of 0.06 N HCl.

### 2.4. MALDI-TOF MS analysis

Following removal from the biosensor, chips were washed with three 200 μl aliquots of distilled water and prepared for MS by application of a MALDI matrix (aqueous solution of α-cyano-4-hydroxycinnamic acid, in 33% (v/v) acetonitrile, 0.2% (v/v) trifluoroacetic acid) with a matrix aerosol application device [20]. The MS analysis was performed on a homemade MALDI-TOF mass spectrometer [9]. Each site/FC on the sensor chip was targeted individually with the nitrogen laser, allowing site-specific BIA/MS analysis. The mass spectra were acquired in positive ion-mode.

## 3. Results and discussion

Following the immobilization of ~111 fmol of anti-IGF-1 in FC2 and 150 fmol of anti-IGF-2 in FC1 (sensorgrams not shown), a 50 μl aliquot of fresh, 50-fold diluted human plasma was injected over the antibody derivatized FC1 and FC2 surfaces (Fig. 1a). At the time of chip undocking from the biosensor, responses of 250 and 164 RU (response unit) were observed in FC1 and FC2, indicating binding of 250 and 164 pg of proteinaceous material, respectively. The mass spectra taken from the surfaces of the two FCs after the plasma injection are shown in Fig. 1b. Noticeable are signals (singly and doubly charged ions) coming from the targeted proteins: IGF-1 signals ($MW_{IGF-1}$ = 7648.7) dominate the spectrum obtained from the surface of FC2 (the anti-IGF-1 derivatized FC), whereas signals from IGF-2 ($MW_{IGF-2}$ = 7469.4) are observed in the mass spectrum taken from the FC1 surface (the anti-IGF-2 derivatized FC). Interestingly, smaller intensity signals from IGF-2 in FC2, and IGF-1 in FC1, are also present, even though they were not targeted by the corresponding antibodies in these FCs. There are three possible explanation for the observance of these signals: (1) an analyte cross-walk occurred between the two FCs in the post-biosensor manipulation (most notably, the application of the MALDI matrix); (2) the immobilized antibodies exhibit cross-reactivity toward the non-targeted protein (as already stated, the IGFs share 62% sequence homology; and (3) a protein complex containing both IGF-1 and IGF-2 was retrieved during the SPR analysis.

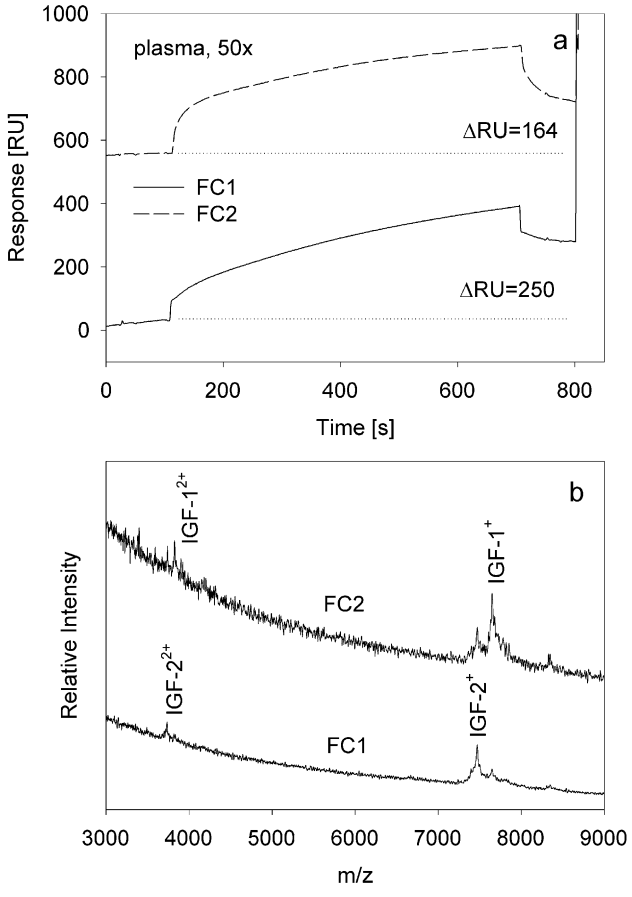


Fig. 1. a: SPR sensorgrams resulting from an injection of 50-fold diluted human plasma sample over the anti-IGF-1 and anti-IGF-2 derivatized FC surfaces. b: MALDI-TOF mass spectra taken from the anti-IGF-1 and anti-IGF-2 derivatized FCs following the injection of the 50-fold diluted plasma sample.

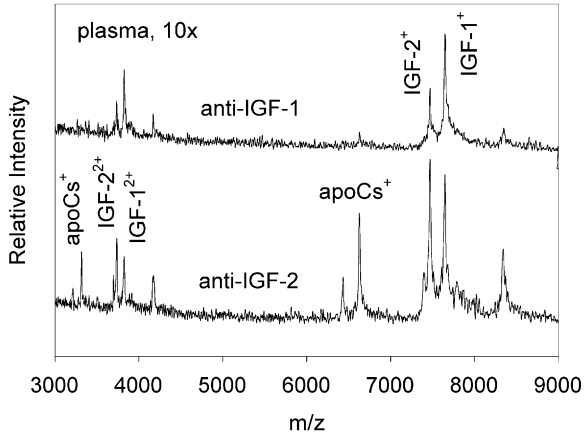


Fig. 2. MALDI-TOF mass spectra taken from anti-IGF-1 and anti-IGF-2 derivatized FCs (on separate sensor chips), following injections of 10-fold diluted plasma samples.

In order to eliminate the possibility of cross-walking between the adjacent FCs, two additional CM5 chips was utilized: a single FC on the first chip was derivatized with anti-IGF-1, and one FC on the second chip was derivatized with anti-IGF-2. A 50 μl aliquot of fresh human plasma, diluted 10-fold, was injected over both chips in two separate experiments (sensorgrams not shown), and the chips were undocked and analyzed via MALDI-TOF MS. The resulting mass spectra are shown in Fig. 2. The presence the two IGFs in both mass spectra is clearly indicated by their corresponding signals, discounting the possibility of FC-cross-walking in the previous experiment. Moreover, due to the better resolution of the spectra, the signal at lower *m/z* from the main IGF-2 peak in the mass spectrum obtained from the anti-IGF-2 derivatized FC was identified as a truncated form of IGF-2 missing its N-terminal Alanine (MW = 7398.3). The spectra also contain several other signals, two of which can be attributed to apolipoprotein C-I (ApoC-I, MW = 6,630.6) and its truncated isoform missing the N-terminal Thr–Pro residues (ApoC-I′, MW = 6432.4). ApoC-I and ApoC-I′ are abundant plasma proteins that, as we have shown previously [20,21],

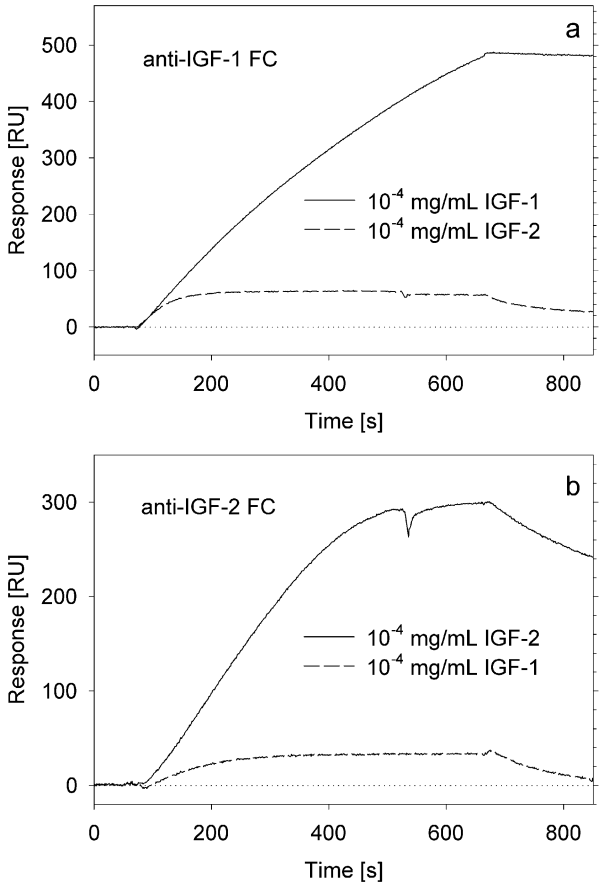


Fig. 3. SPR sensorgrams showing the injections of purified solution of recombinant IGF-1 and IGF-2 over anti-IGF-1 (a) and anti-IGF-2 (b) derivatized FCs. The sensorgrams shown were baseline corrected by mathematically subtracting buffer (HBS-EP) curves (obtained via injections of 50 μl aliquots of HBS-EP buffer right before the samples injections, not shown in the figure) from the samples curves.

bind non-specifically to the chip surface. This higher level of non-specific binding was somewhat expected due to the high concentration of plasma (10-fold) utilized in this example.

To delineate the antibodies cross-reactivity, another CM5 chip was derivatized with anti-IGF-1 and anti-IGF-2 antibodies. This time, instead of plasma, pure samples of IGF-1 and IGF-2 (recombinant, and extensively purified, >98%, in HBS-EP buffer) were injected over the surfaces at concentrations of $10^{-3}$, $10^{-4}$ and $10^{-5}$ mg/ml. The resulting sensorgrams were baseline corrected by mathematically subtracting buffer (HBS-EP) curves (obtained via injections of 50 μl aliquots of HBS-EP buffer right before the samples injections[1]) from the samples curves. Shown in Fig. 3 are the sensorgrams resulting from the injections of 50 μl aliquots of $10^{-4}$ mg/ml solutions of IGF-1 and IGF-2 over anti-IGF-1 and anti-IGF-2 derivatized FCs (the normal levels of IGF-1 and IGF-2 in plasma are ~150 μg/ml and ~500 μg/ml, respectively [22]). The SPR responses taken 180 s after the end of the injections indicate that the amount of cross-reactivity for each of the antibodies was less than 5%, which can not account for the relative ratios of the IGF-1 and IGF-2 signals observed in Fig. 2 (similar cross-reactivities were observed for the other two concentrations studied, results not shown). To ultimately test the antibodies' cross-reactivities, a 50 μl aliquot of HBS-EP solution containing both IGF-1 and IGF-2 (each at $10^{-4}$ mg/ml) was injected over the anti-IGF-1 and anti-IGF-2 antibody derivatized surfaces and the chip was subjected to MALDI-TOF MS. The resulting mass spectra (Fig. 4) indicate that only IGF-1 and IGF-2 were retrieved in their corresponding FC, without any cross-reactivities.

In another set of control experiments, recombinant IGF-1 and IGF-2 were immobilized on the sensor chip surfaces and screened for possible interactions with each other via injections of pure IGFs samples (results not shown). As expected, no such interaction was detected. However, the interactions of the IGFs with IGFBPs have been extensively documented [23–25] and it possible that the immobilized antibodies retrieved the entire IGF–IGFBPs complex, which, upon matrix application, fell apart and yielded the IGF-1 and IGF-2 signals in the mass spectra. Consequently, signals from the IGFBP should also have been observed in the mass spectra shown in Fig. 2. However, other than several unspecified wide peaks, and a relatively noisy baseline, signals due to specific IGFBPs could not be explicitly delineated in the higher mass region (not shown).

In order to more substantially demonstrate the retrieval of the protein complex, fresh human plasma was treated with several detergents to possibly disrupt the protein complex and release its constituent proteins. For the first sample, 20 μl of pure plasma (undiluted) was mixed with 20 μl of 0.5% SDS solution, incubated 30 min at room temperature, and further diluted with 160 μl of HBS-EP buffer to yield a plasma sample diluted 10-fold in buffer and 0.05% SDS. Another sample of plasma (10-fold diluted) was prepared in HBS-EP buffer containing 0.1% Tween 20. These two samples, along with a non-treated plasma control sample (10-fold diluted in HBS-EP) were injected in 10 μl aliquots over anti-IGF-1 and IGF-2 derivatized surfaces on a new CM5 sensor chip. The

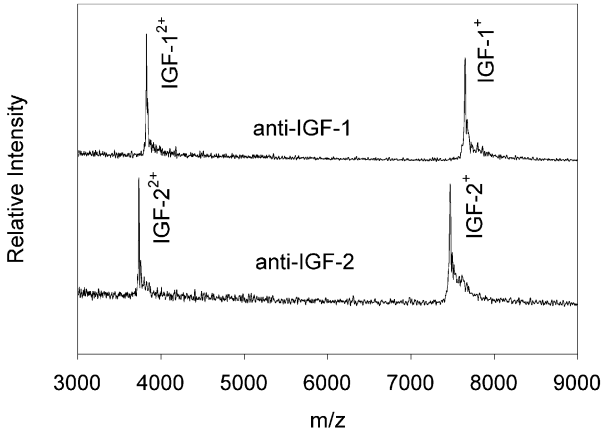


Fig. 4. MALDI-TOF mass spectra taken from anti-IGF-1 and anti-IGF-2 derivatized FCs following an injection of a buffer solution containing $10^{-4}$ mg/ml IGF-1 and IGF-2.

[1] This type of SPR signal referencing was necessary due to the constant positive bulk refractive index change observed during the SPR analysis, which was different between the two FCs, thus eliminating the possibility for parallel FC referencing. Also, to make the SPR readings consistent, all injections were performed exactly 60 s following the end of the regeneration solution (0.06 M HCl) injection, and readings taken 180 s after the end of the injections, which were performed without the consequent wash.

resulting sensorgrams are shown in Fig. 5. The injection of the SDS-treated plasma sample resulted in SPR responses of 80 and 51 RU in FC1 and FC2, respectively (the readings were taken 85 s after the end of the injections). These responses are significantly lower than the responses observed from the untreated sample injection (288 RU in FC1 and 197 RU in FC2), and the SPR responses observed after the injection of the Tween-treated plasma sample (239 RU in FC1 and 246 in FC2). The lower responses observed for the SDS-plasma sample could indicate the possible disruption of the protein complex and retrieval of only IGF-1 and IGF-2 by the immobilized antibodies, which would in turn yield lower SPR responses due to the lesser amount of total protein amount captured on the surface. In preparation for MALDI-TOF MS analysis, another aliquot of the SDS-treated plasma sample (50 μl) was injected over the regenerated surface of the same sensor chip, yielding SPR responses of 287 and 96 RU in FC1 and FC2, respectively (sensorgram not shown). The mass spectra taken from the surface of this sensor chip are shown in Fig. 6. The signals from the targeted proteins (IGF-1 in the anti-IGF-1 derivatized FC, and IGF-2 and its truncated isoform in the anti-IGF-2 FC) dominate the spectra, with very little presence of the other non-targeted IGF. The peak ratio of targeted vs. non-targeted IGFs is clearly different from the one observed in Fig. 2, providing a strong evidence that the treatment of the plasma sample with SDS released the IGFs from a putative protein complex. The spectra shown in Fig. 6

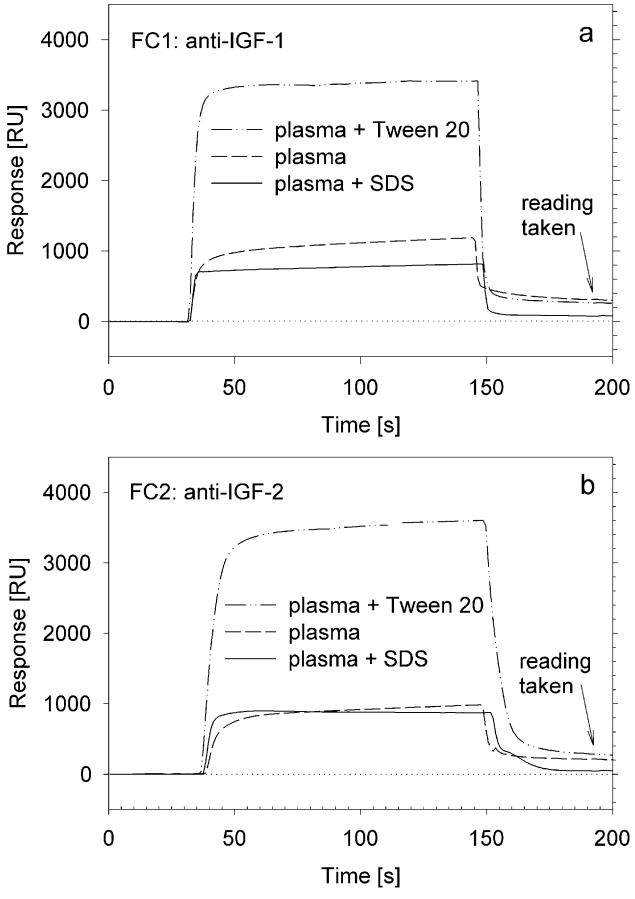


Fig. 5. SPR sensorgrams resulting from injections of 10-fold diluted pure, Tween-treated, and SDS-treated plasma samples, over anti-IGF-1 (a) and anti-IGF-2 (b) derivatized FCs.

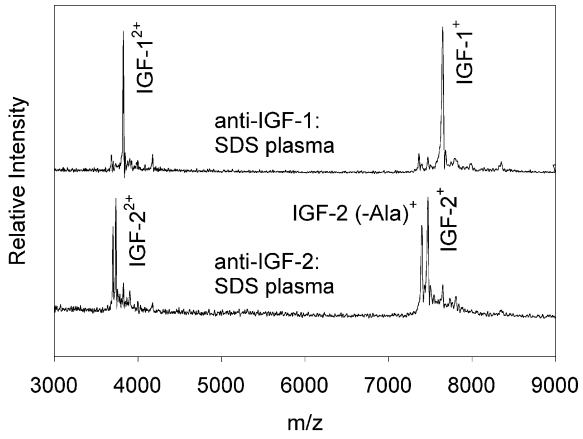


Fig. 6. MALDI-TOF mass spectra taken from the anti-IGF-1 and anti-IGF-2 derivatized FC surfaces following the injection of the SDS-treated plasma sample.

were much easier to obtain (with better *S*/*N* ratio and resolution) than those shown in Fig. 2, adding to the argument that there were more components that just the targeted proteins retrieved on the surface of the sensor chip in the experiments leading to Fig. 2.

The experimental data shown in this work suggest that both bound and free IGF-1 and IGF-2 from human plasma were detected by using single antibodies but different sample preparation. Several control experiments were performed to ensure the validity of the data. Most importantly, it was established that the antibodies do not exhibit significant cross-reactivity toward the other, non-targeted IGF. The only possible explanation for the retrieval of both IGFs from plasma under native conditions is that the proteins are joined together in a multi-protein complex. Whereas it is a well established that IGF-1 and -2 do not interact with each other (which was also verified in a control experiment in this study), it is known that most of the circulating IGF-1 and IGF-2 are complexed to IGFBPs. For this reason, we postulate that the entire IGF–IGFBP protein complex was retrieved by the antibodies under native conditions, yielding signals from both IGFs in the mass spectra. The absence of signals from IGFBPs in the mass spectra might be attributed to signal suppression effects, which often occur when one protein yields strong signals in the mass spectrum so that signals of other proteins present in the sample are suppressed and, consequently, not observed. Small proteins (such as the IGFs) are known to ionize readily and much easier than higher MW proteins (such as the IGFBP3, the major IGFBP, with three possible glycosylation sites), yielding to suppression of the higher MW signals.

In summary, ligands with affinities toward a protein that is part of in-vivo assembled complexes can be used as 'hooks' to retrieve the entire protein complex from a biological sample prepared under native (non-denaturing) conditions. In BIA/MS, the SPR sensing offers a unique opportunity to monitor the state of these protein complexes as a function of solvent variations, whereas the subsequent MALDI-TOF MS analysis of the retained components yields signals that reveal the masses of the constituent proteins, along with any structural modifications present. Given the dual aspect of the analysis (quantitative and qualitative), BIA/MS holds great promise in investigating protein complexes and the mechanisms behind their assembly.

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