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Authors

Ouimet, Claire M
Dawod, Mohamed
Grinias, James
et al.

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Protein Cross-linking Capillary Electrophoresis at Increased Throughput for a Range of Protein-Protein Interactions

Claire M. Ouimet[a], **Mohamed Dawod**[a], **James Grinias**[a,b], **Victoria A. Assimon**[c], **Jean Lodge**[d], **Anna K. Mapp**[a,d], **Jason E. Gestwicki**[c], and **Robert T. Kennedy**[a,e,*]

[a]Department of Chemistry, University of Michigan, Ann Arbor, MI, 48109, United States

[b]Department of Chemistry, Rowan University, Glassboro, NJ 08028, United States

[c]Department of Pharmaceutical Chemistry, University of California at San Francisco, California 94158, United States

[d]Program in Chemical Biology, University of Michigan, Ann Arbor, MI 48109, United States

[e]Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109, United States

Abstract

Tools for measuring affinities and stoichiometries of protein-protein complexes are valuable for elucidating the role of protein-protein interactions (PPIs) in governing cell functions and screening for PPI modulators. Such measurements can be challenging because PPIs can span a wide range of affinities and include stoichiometries from dimers to high order oligomers. Also, most techniques require large amounts of protein which can hamper research for difficult to obtain proteins. Protein cross-linking capillary electrophoresis (PXCE) has the potential to directly measure PPIs and even resolve multiple PPIs while consuming attomole quantities. Previously PXCE has only been used for high affinity, 1:1 complexes; here we expand the utility of PXCE to access a wide range of PPIs including weak and multimeric oligomers. Use of glutaraldehyde as the cross-linking agent was key to advancing the method because of its rapid reaction kinetics. A 10 s reaction time was found to be sufficient for cross-linking and quantification of seven different PPIs with K_d values ranging from low μM to low nM including heat shock protein 70 (Hsp70) interacting with heat shock organizing protein (3.8 ± 0.7 μM) and bcl2 associated anthanogene (26 ± 6 nM). Non-specific cross-linking of protein aggregates was found to be minimal at protein concentrations < 20 μM as assessed by size exclusion chromatography. PXCE was sensitive enough to measure changes in PPI affinity induced by the protein nucleotide state or point mutations in the protein-binding site. Further, several interactions could be resolved in a single run, including Hsp70 monomer, homodimer and Hsp70 complexed the with c-terminus of Hsp70 interacting protein (CHIP). Finally, the throughput of PXCE was increased to 1 min per sample suggesting potential for utility in screening.

*Corresponding author: rtkenn@umich.edu, Tel: 734-615-4363, Fax: 745-615-6462.

Conflicts of interest

There are no conflicts to declare.

Introduction

Many proteins participate in protein-protein interactions (PPIs) of varying affinities and complexities. Although a variety of methods for PPI analysis exist, most have limitations such as requiring large amounts of protein sample, utility only in a narrow affinity range, or suitability for analysis of only one interaction at a time. Affinity Probe Capillary Electrophoresis (APCE) has the potential to overcome these limitations and become a useful tool for measurement and screening of PPIs. In APCE, one of the binding partners is labeled with a fluorophore, pre-equilibrated with binding partner, and then analyzed by capillary electrophoresis with laser induced fluorescence detection (CE-LIF) so that free and bound proteins can be detected.[1] APCE is an attractive platform for addressing challenges in PPI analysis as sample requirements are low, complexes of varying affinity are tractable, and the separation component provides resolution between different complexes.

APCE method development for PPIs can be difficult if electrophoretic mobility changes upon protein binding are small, proteins adsorb to the capillary wall, or complexes dissociate during the separation. Protein adsorption causes irreproducibility and distorts peak shapes and areas preventing accurate quantification. Conditions that mitigate adsorption, such as extreme pH or SDS-denaturation with sieving separation, are not generally compatible with native protein interactions. Complex dissociation during separation is problematic for transient interactions where complex dissociation occurs on the timescale of the separation. Identification of conditions that are suitable for separation and maintenance of multiple interactions is even more challenging. Quantification of multiple peptides binding to the same protein has been reported by CE;[2] however, APCE of multimeric protein-protein complexes has not been reported. As a result of these challenges, APCE is not yet a widely used method for analysis of PPIs.

Previously we reported a modified APCE method, termed protein cross-linking capillary electrophoresis (PXCE), which simplified method development and may be more generally applicable for PPI analysis. In this method, interacting proteins are covalently cross-linked prior to electrophoresis. By decoupling the binding reaction and separation step, it is possible to use harsh separation conditions, such as denaturing capillary gel electrophoresis (CGE), which can reliably resolve protein complexes based on size using standard conditions. The original report of PXCE used cross-linking with formaldehyde for 10 min and was demonstrated for analysis of dimeric PPIs with low nanomolar K_d values.[3] As reported here, these conditions are limited to high affinity PPIs. An ideal method would allow for the quantification of low to high affinity PPIs as well as complexes that consist of higher order oligomers. Also, the method required at least 10 min for gel separation, limiting its throughput.

Here we report a PXCE method capable of quantitative analysis of PPIs with low nM to low μM K_d values by using glutaraldehyde as the cross-linker, a reagent previously demonstrated to have rapid reaction kinetics and high yields.[4,5] We also demonstrate higher throughput CGE through use of a lower viscosity separation media and overlapping injections so that individual assays can be completed in 1 min. Finally we demonstrate the potential for analyzing multimeric complexes. The method is demonstrated on an array of chaperone and

transcription factor proteins including heat shock protein 70 (Hsp70) homodimer, Hsp70-heat shock organizing protein (HOP), Hsp70-bcl2 association athanogene 3 (Bag3), heat shock protein 90 (Hsp90) homodimer, Hsp70-c-terminus of Hsp70 interacting protein (CHIP), KIX domain of CREB-binding protein (KIX)-E2A17, and KIX-c-Myb.

Experimental

Materials and Reagents

All reagents were purchased from Millipore Sigma (St Louis, MO), unless otherwise specified. All buffers were made using 18 MΩ water deionized by a Series 1090 E-pure system (Barnstead Thermolyne, Dubuque, IA).

Protein Purification and Labeling

Hsp70 and Hsp90 were purified and labeled with AlexaFluor488 as previously described.[3,6] Recombinant human CHIP, HOP, and HOP mutants were purified as described previously.[7] The peptides c-Myb and E2A17 were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) solid phase synthesis with CLEAR-Amide Resin (Peptides International, Inc.). c-Myb (FITC-βAla-KEKRIKELELLLMSTENELKGQQALW-NH_2) and E2A17 (FITC-βAla-GTDKELSDLLDFSAMFS-$CONH_2$) were FITC labeled, dried under nitrogen, precipitated, washed with cold ether, purified by reverse phase HPLC, lyophilized and dissolved in DMSO. The KIX domain of mouse CREB-binding protein was expressed and purified as previously described,[8] for a resulting molecular weight of 12 kDa. Protein molecular weights are included in Table S1.

Protein Cross-linking

Proteins were allowed to equilibrate in assay buffer at room temperature for at least 30 min prior to cross-linking. For nucleotide state dependence experiments proteins were incubated with either 10 mM ATP or 10 mM ADP. For KIX-c-Myb and KIX-E2A17 interaction the assay buffer was 10 mM sodium phosphate, 100 mM NaCl, pH 6.8. For Hsp70-HOP the assay buffer was 50 mM HEPES, 75 mM NaCl, 0.001% Triton X pH 7.4. For all other PPIs an assay buffer of 25 mM HEPES, 10 mM KCl, and 5 mM $MgCl_2$, pH 7.5, was utilized. Cross-linking was carried out at room temperature by a 1:20 dilution of 40% w/v glutaraldehyde to a final concentration of 2% w/v glutaraldehyde, unless otherwise specified. The cross-linking reaction was quenched with 800 mM Tris after 10 s for a final concentration of 400 mM Tris unless otherwise stated. Lastly, SDS was added prior to injection to a final concentration of 0.2% w/v for sieving separations.

Capillary Electrophoresis

A Beckman Coulter P/ACE MDQ Capillary Electrophoresis instrument (Fullerton, CA) was used for all experiments. The excitation wavelength filter was 488 nm and the emission wavelength filter was 520 nm. Fused silica capillary (Polymicro Technologies; Phoenix, AZ) was 360 μm outer diameter 40 μm inner diameter unless otherwise stated. The total capillary length was 30 cm and the capillary length to detector was 10 cm. Electropherograms were acquired using 32 Karat software (Beckman Coulter), were analyzed using Cutter 7.0 software,[9] and data were fit using GraphPad Prism 7.

For PXCE separations the sieving media was 180 mM tris, 200 mM borate, 1 mM EDTA, 13.8 mM SDS, 7% dextran (1,500-2,800 kDa), and 10% glycerol unless otherwise specified. Samples were introduced by hydrodynamic injection at 5 psi for 15 s. A field of 567 V/cm was applied for separation. Capillaries were preconditioned by sequentially rinsing with water, hydrochloric acid, water, sodium hydroxide, water, UltraTrol LN (Target Discovery, Palo Alto, CA), and finally gel media. Capillaries were reconditioned after a noticeable shift in migration time was observed, typically after one hour of continuous operation.

For affinity probe capillary electrophoresis (APCE) without cross-linking the capillary was conditioned sequentially with sodium hydroxide, water, and run buffer. The running buffer was 25 mM HEPES, pH 7.5. Samples were introduced electrokinetically with an applied voltage of 5 kV for 5 s. The separation field was 1,000 V/cm. The capillary was reconditioned between injections with sodium hydroxide followed by running buffer.

For increased throughput PXCE, the sieving media was 90 mM Tris, 100 mM borate, 1 mM EDTA, 13.8 mM SDS, 3.5% dextran (1,500-2,800 kDa), 5% sorbitol and a 25 μm inner diameter 360 μm outer diameter capillary with 30 cm total length and 10 cm to detector was used. Samples were introduced sequentially by hydrodynamic injection for 15 s followed by separation at 1 kV/cm.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) was carried out on an Agilent 1100 (Santa Clara, CA) using a TSKgel SuperSW3000 column (Millipore Sigma) with a flow rate of 0.3 mL/min and a sample injection volume of 1 μL. Detection was by UV absorbance at 280 nm. The mobile phase was 0.05% sodium azide, 0.1 M sodium sulfate, 0.1 M sodium phosphate, pH 6.7. Data were sampled at 20 Hz with a 14-bit data acquisition card (National Instruments, Austin, TX). Chromatograms were acquired using an in-house LabView (National Instruments, Austin, TX) program.

Results and discussion

Cross-linking Conditions and Characterization of Non-specific Interactions

It has been reported that for highly dynamic interactions, formaldehyde cross-linking is not effective because of an insufficiently fast reaction under certain conditions.[10] If the cross-linking reaction kinetics are not much faster than the dissociation kinetics, then the fraction of complex cross-linked will be lower than that present under equilibrium conditions.[11] Glutaraldehyde reacts rapidly at high cross-linker concentration[12] which is expected to minimize under-cross-linking, even of transient complexes[5]. We hypothesized that such a reaction might be better able to capture complexes for a PXCE experiment. We tested the hypothesis that glutaraldehyde would be a sufficient cross-linker for the highly dynamic interaction between Hsp70 and HOP which has previously been observed to have fast dissociation and association kinetics.[13,14] We found 10 min of formaldehyde cross-linking was insufficient to covalently capture this interaction; however, a 10 s reaction with glutaraldehyde led to a large amount of complex being detected (Figure 1A,B).

We then tested the effect of glutaraldehyde concentration and reaction time on yield of complex for three interactions with K_d values in the low μM range: Hsp70 homodimer, Hsp70-HOP and c-Myb-KIX. These results showed that the highest yields of covalent complex were achieved with less than 1 min of cross-linking with 2% w/v glutaraldehyde. Interestingly, for both Hsp70-HOP and c-Myb-KIX, reactions as short as 10 s were sufficient for the highest yields of cross-linked product (Figure 1D,E).

To further investigate the effects of cross-linking and the conditions under which specific interactions are favored over nonspecific interactions, SEC was employed. SEC mobile phase is sometimes compatible with native PPI conditions and, as a result, complexes can be observed without cross-linking. The high mass range of SEC also allows for high molecular weight aggregates resulting from nonspecific cross-linking to be monitored. Bag3 and HOP were chosen as model proteins to determine the cross-linking reaction conditions that result in non-specific aggregation. Bag3 and HOP are not expected to self-associate into high mass complexes[15,16] and minimal aggregation was observed by SEC without cross-linking even at high protein concentration. With a 10 s cross-linking reaction however, higher molecular weight species, presumably aggregates, were detectable at protein concentrations of 20 μM and above (Figure S1A). This result suggests an upper limit to protein concentrations that can be studied using this method. Non-specific interactions are expected to dominate in crowded protein solutions as the probability of non-interacting proteins becoming cross-linked increases. For example, with NHS ester cross-linking, non-specific complexes were observed at protein concentrations at 40 μM[11] and at 33 μM[17].

Besides high protein concentrations, we also considered the possibility that long cross-linking times could lead to additional aggregation. It has previously been reported that glutaraldehyde cross-linking is specific only at short reaction times[18]. At 10 μM, Bag3 and HOP were not found to aggregate even at long cross-linking reaction times. Hsp70, however, was found to exhibit increasing aggregation by SEC at cross-linking times greater than about ~70 s (Figure S1B). Slow, higher order aggregation of Hsp70 has been observed previously[19,20], potentially explaining the increase seen at long cross-linking times. This result explains previous observations of decreasing yields of complex at long cross-linking reaction times with formaldehyde cross-linking PXCE.[3] That is, higher molecular weight aggregates are preferentially excluded from the gel during injection this preventing detection and the result is observation of low complex formation. This effect likely explains the apparent drop in complex yield in the PXCE experiments at longer reaction times (600 s) in Figure 1C,D. To minimize the effects of non-specific or additional high mass aggregation, we limited our assays to total protein concentrations of 20 μM or less with cross-linking reaction times of 10 s. These conditions circumscribe the assay to PPIs with low μM or lower K_ds where saturation binding experiments can be completed without using overly high concentrations of protein, circumventing the need to subtract nonspecific cross-linking to determine binding affinity.

Determination of PPI Affinity

We next determined if 10 s reactions with proteins below 20 μM would be sufficient to allow for quantification of binding affinity for a variety of PPIs. For these experiments, saturation

binding curves were carried out by PXCE on PPIs with low μM to low nM K_d values (Figure 2). Good agreement was found between the K_d values determined by PXCE and values reported by non-cross-linking methods for Hsp70-Bag3, Hsp90 homodimer, Hsp70 homodimer, Hsp70-HOP, and KIX-c-Myb (Figure 3, Table S2). We conclude that PXCE with 10 s glutaraldehyde cross-linking time is sufficient for measuring the affinities of these complexes for K_d values spanning three orders of magnitude from low nM to low μM values.

Sensitivity of Assay to Point Mutations and Nucleotide State

We next determined the sensitivity of the glutaraldehyde PXCE method to changes in protein structure by measuring the effect of binding site point mutants and the nucleotide state on interaction affinity. Hsp70 is an ATPase known to undergo nucleotide state dependent structural changes leading to a changes in the degree of homo-oligomerization.[19,21] Here, clear differences in homodimerization affinity were seen for Hsp70 with the ATP state having the weakest affinity and the ADP state having the greatest affinity for homodimerizing (Figure 4). The sensitivity of the PXCE assay to such structural changes lends further evidence to the specificity of this method.

HOP proteins with one-site substitution mutations in the Hsp70 binding site were also investigated. HOP with mutations R77A and N223A inhibit the interaction of HOP with Hsp70.[7] Using a 10 s glutaraldehyde cross-linking reaction the wild type protein had a lower K_d value than the mutants, as expected (Figure 5). In fact, the rank order for the K_d values of the two mutants was in agreement between PXCE and values obtained using fluorescence polarization[7]. The R77A mutant HOP however, was found to have a much higher K_d value by fluorescence polarization (>25 μM) than by PXCE (3 ± 1 μM), indicative of a much lower apparent affinity for Hsp70 by fluorescence polarization compared to PXCE. Notably, the PXCE assay was carried out on full length proteins while the fluorescence polarization assay was of the interaction between full length HOP and a peptide consisting of the EEVD domain of Hsp70.

Two potential explanations for the discrepancy in K_d values between the fluorescence polarization and PXCE assays were explored: 1) The PXCE K_d is an artifact of cross-linking and 2) Full length Hsp70 has a higher affinity for HOPR77A than does the EEVD domain alone. The affinity of full length Hsp70 for HOPR77A was confirmed by APCE without cross-linking (K_d = 4 ± 2 μM, Figure S2) suggesting that the K_d measured by PXCE was not a cross-linking artifact. To compare the affinities of full length Hsp70 and the EEVD domain alone for HOPR77A a competitive binding experiment was carried out (Figure S3). Full length Hsp70 was found to have higher affinity for HOPR77A than either the EEVD domain or the full-length form of Hsp70 with a deletion in the EEVD domain. This result is in agreement with other reports which have found the EEVD domain to be important for the Hsp70-HOP interaction while not accounting for all of the affinity.[7,14] Compatibility with full length protein is an advantage of this CE assay as use of full length protein eliminates the method development or artifacts associated with use of peptide necessary for fluorescence polarization.

Increased Throughput Electrophoretic Sieving Separation

In view of the apparent utility of the method for measuring affinities for a wide range of PPIs, we sought to improve the throughput of the method. Although microchip CE and other techniques can achieve separations in seconds, in this case we used a commercial CE instrument to maintain accessibility to a wider range of potential users. Decreasing the viscosity of the sieving media was seen as a key to improving throughput because it both allows faster migration at a given electric field and allows smaller bore capillaries to be used. Higher fields can be applied without detrimental heating effects by use of lower diameter capillary; however, with viscous sieving matrix it can be difficult to rinse the matrix through small bore capillaries. Use of ultralow viscosity sieving matrices have been reported for DNA separations[22,23] and are compatible with rapid capillary regeneration. Here, an ultralow viscosity sieving matrix (7.45 ± 0.05 cP) was utilized for protein separation. This matrix is about 1/5th as viscous as the matrix used above however sufficient separation of 11-155 kDa proteins was maintained (Figure S4). The ultralow viscosity matrix can be rinsed through a 25 μm capillary in 5 min (at 50 psi > 3 capillary volumes are replaced) while the higher viscosity matrix would require 26 min to be rinsed through a 25 μm capillary. Using the ultralow viscosity matrix, a 1 kV/cm field can be applied in a 25 μm capillary without observing the heating effects that were observed in 40 μm capillary (Figure S5). This high field allows for 11-155 kDa proteins to be separated in 2 min (Figure S4).

It is possible to further increase the throughput of sequential injections by overlapping the injections based on the separation window and the time that occurs prior to the first peak in the separation.[24] By using overlapping injections on a 25 μm capillary with the ultralow viscosity sieving matrix it was possible to make sequential injections and separations with a throughput of 1 sample/min (Figure 6). The samples could be repeatedly injected for up to about 70 min before the capillary needed rinsing. Saturation binding curve data was obtained from this electropherogram and a K_d for the binding interaction was determined to be 1.1 ± 0.2 μM (Figure 6C), in good agreement with the K_d value obtained by fluorescence polarization of 1.1 ± 0.2 μM. This strategy could also be used for determination of small molecule PPI inhibitor potency. A dose response curve for Hsp70-Bag3 inhibitor JG-98 was carried out using overlapping injection for 8 samples in 10 min for a single replicate. A log(IC_{50}) value of -6.4 ± 0.1 was determined (Figure 6F) which is in good agreement with the previously reported log(IC_{50}) value of -6.1 ± 0.3 showing good accuracy of the method.[3]

Application to Higher Order Oligomers

Many of the proteins discussed here form additional higher order or multi-protein complexes. To determine the utility of this method for application to higher order oligomers, the binding of Hsp70 to CHIP was investigated. At 0.5 μM Hsp70 in the apo nucleotide state both monomeric and dimeric Hsp70 are observed. Upon addition of 1 μM CHIP a 2:2 Hsp70:CHIP complex was evident (Figure 7). Previous reports of Hsp70-CHIP complex have found similar results with CHIP forming a homodimer and binding to two Hsp70 to form a 210 kDa complex.[25,26] This result demonstrates the potential utility of this method for the investigation of multimeric complexes. Most methods for PPI analysis, such as isothermal calorimetry (ITC) and fluorescence polarization, are incompatible with resolving multiple interactions in a single assay.

Conclusions

With a fast (10 s) cross-linking reaction, protein complexes with K_d values spanning 3-orders of magnitude, from low nM to low μM, could be quantified by PXCE. The 10 s cross-linking reaction is 60-fold faster than the previous cross-linker used for PXCE. The presence of possible non-specific aggregation at high protein concentrations limits the practical applicability of this method to investigations of low μM or lower K_d values. The molecular weight range of the gel matrix used also limits the method to complexes less than 250 kDa. There is potential to further tune the size limit of the gel as has been demonstrated for other entangled polymer gel matrices.[27]

This method has a number of advantages over other PPI analysis methods. PXCE is compatible with full-length proteins eliminating the need to prepare short peptide mimics of the protein, as is often necessary in fluorescence polarization assays. The ability to distinguish multiple complexes suggests that this method will be useful for evaluating the specificity of a PPI inhibitor as has previously been demonstrated for protein-peptide interactions[2]. By using overlapping injection methods the separation time can be decreased to about 1 min per sample suggesting potential utility of the method when higher throughput is necessary. Dissociation constants and IC_{50} values can each be determined in about 10 min compared to hours required by other methods such as ITC. There is potential for further increasing throughput by using 96- or 384-capillary arrays or microchip electrophoresis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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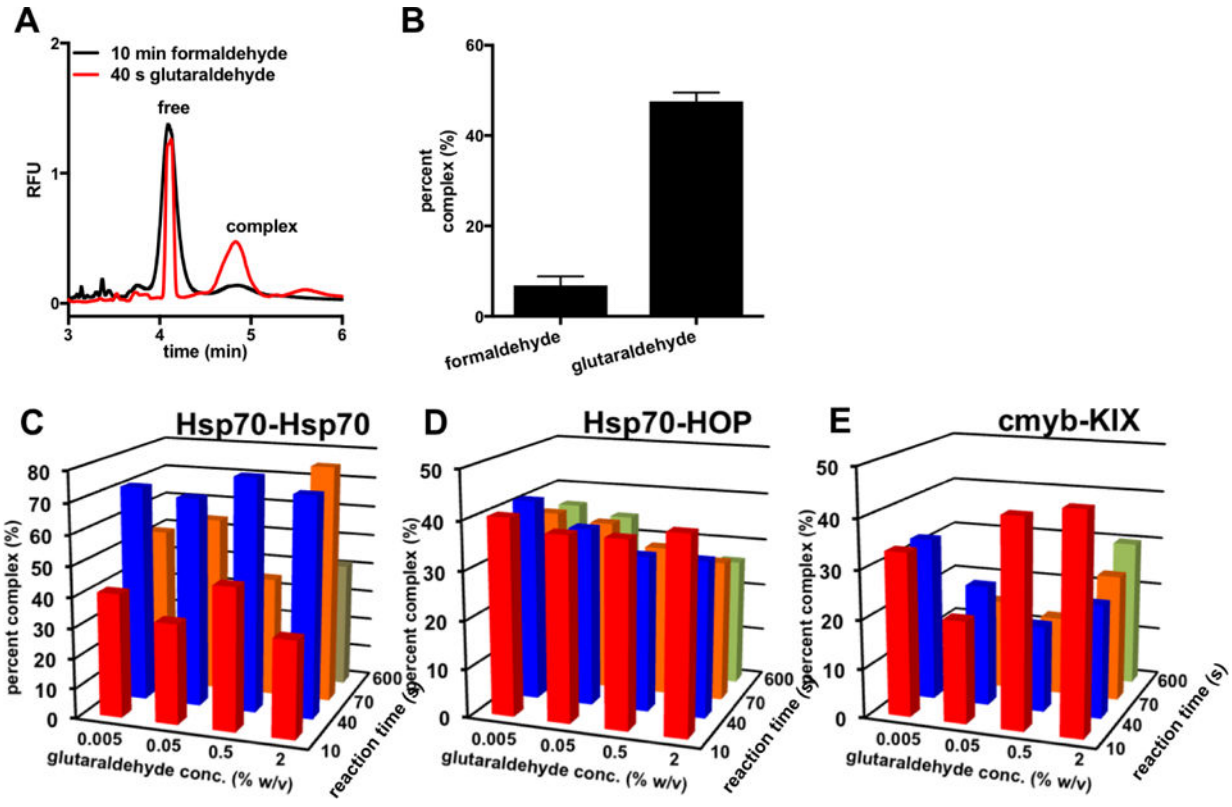

Figure 1.

Evaluation of glutaraldehyde cross-linking yield for low affinity PPIs. Comparison of yield of Hsp70-HOP complex with glutaraldehyde or paraformaldehyde cross-linking reactions (A). Dependence of Hsp70 homodimer (B), Hsp70-HOP (C), and c-Myb-KIX (D) complex yields on glutaraldehyde cross-linking time and concentration.

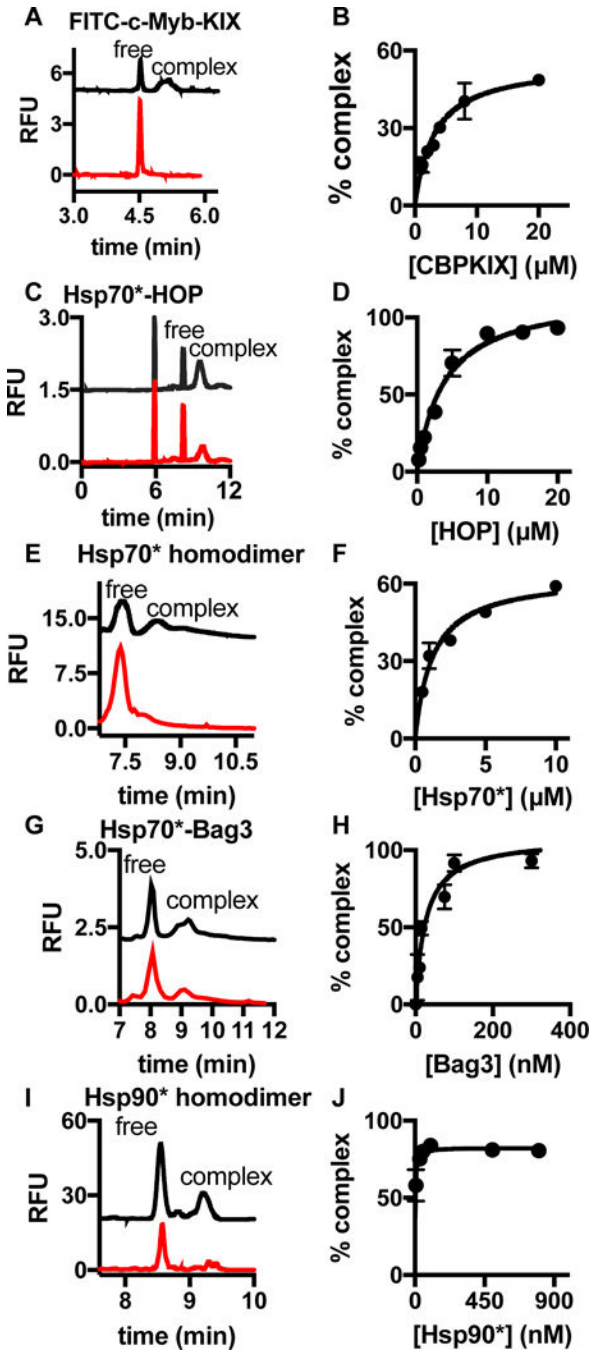

Figure 2.

Electropherograms and saturation binding curves for μM to nM affinity complexes by 10 s glutaraldehyde cross-linking PXCE. Saturation binding curves were fit by nonlinear regression to find K_d = 3.1 ± 0.5 μM, 3.8 ± 0.7 μM, 1.2 ± 0.3 μM, 26 ± 6 nM, 2.1 ± 0.3 nM for FITC-c-Myb-KIX (A), Hsp70*-HOP (B), Hsp70* homodimer (C), Hsp70*-Bag3 (D), and Hsp90* homodimer (E), respectively. The first peak in the Hsp70* electropherograms corresponds to a low molecular weight impurity.

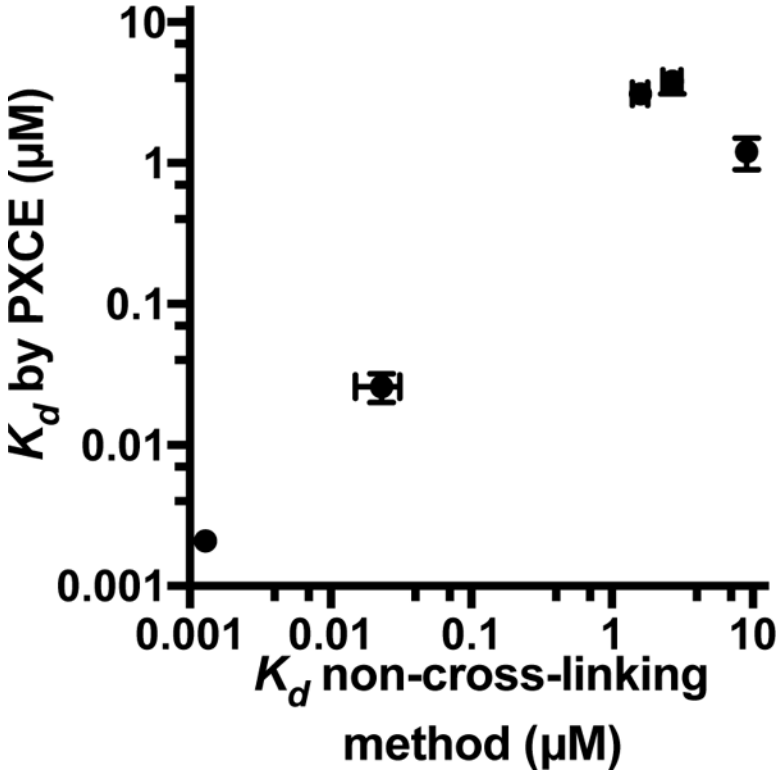

Figure 3.

Comparison of PXCE K_d values from data in Figure 2 to K_d values from non-cross-linking methods for Hsp90 homodimer[28], Hsp70-Bag3[6], Hsp70 homodimer[19], Hsp70-HOP[7], and FITC-c-Myb-KIX.

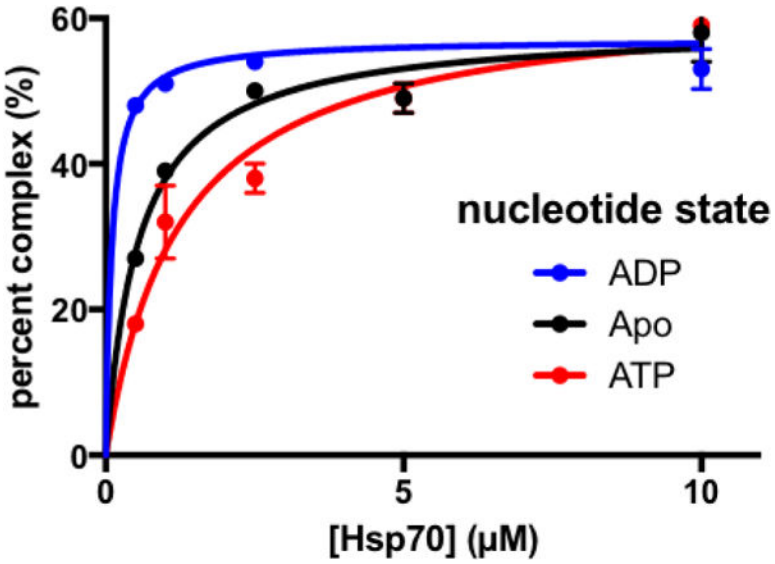

Figure 4.

Sensitivity of 10 s glutaraldehyde cross-linking assay to nucleotide dependent homodimerization of Hsp70*.

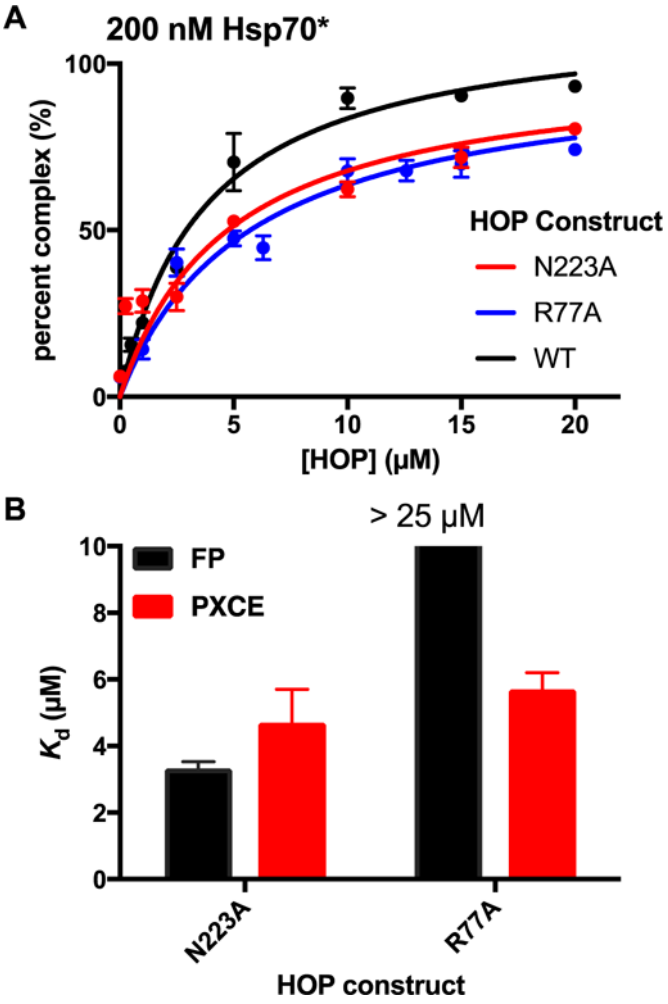

Figure 5.

Sensitivity of 10 s glutaraldehyde cross-linking assay to point mutants of HOP. Saturation binding curves (A) and comparison to APCE and fluorescence polarization[7] data (B).

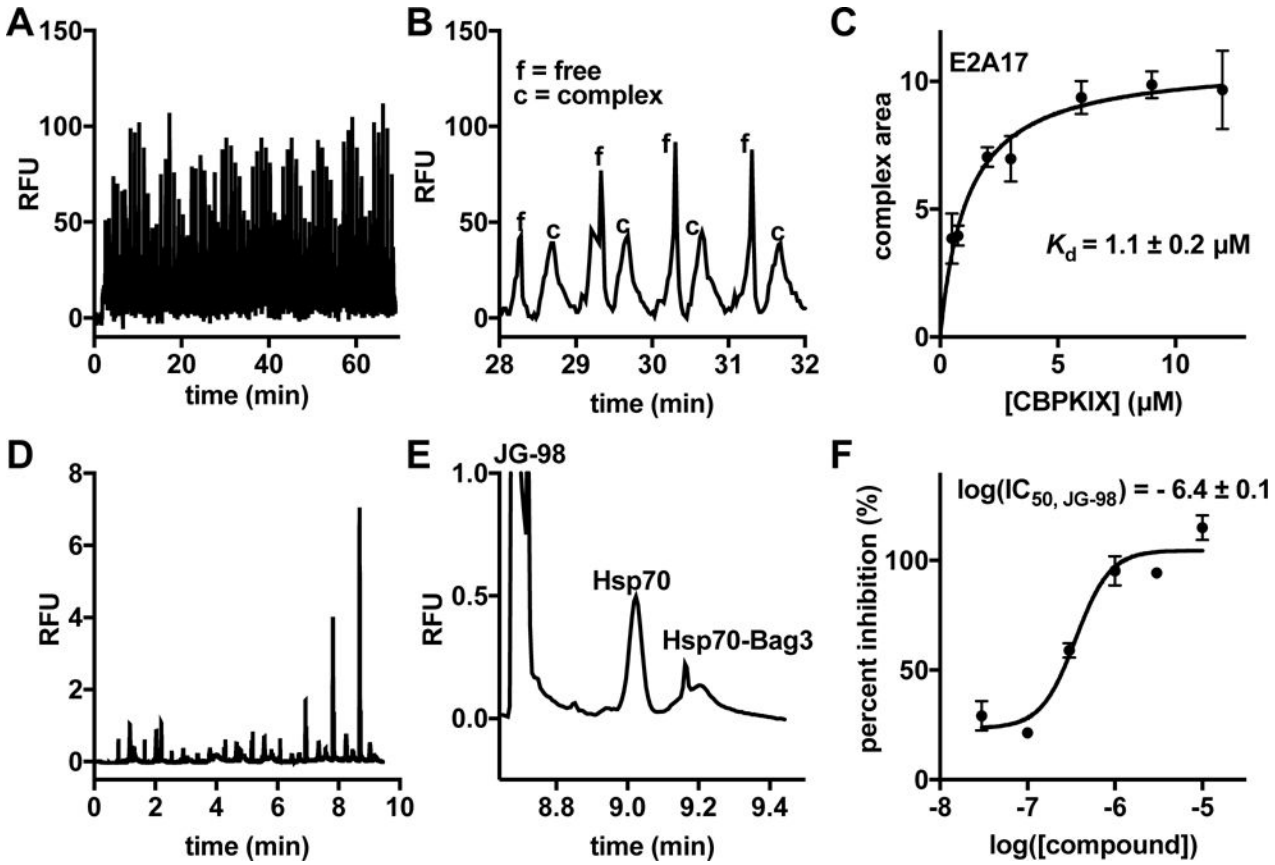

Figure 6.

Overlapping injections for saturation binding curve and IC_{50} determination at increased throughput. Electropherogram of continuous overlapping injections of samples at various KIX concentrations (A). A section from 28 to 32 min of the electropherogram with analytes from injection of four different samples (B). Determination of the K_d value for the E2A17-KIX interaction from the electropherogram shown in A (C). Electropherogram of continuous overlapping injections of Hsp70-Bag3 and fluorescent small molecule inhibitor JG-98 (D). A section of the electropherograms corresponding to analytesfrom one injection. Determination of IC_{50} for Hsp70-Bag3 complex inhibition by JG-98 (F).

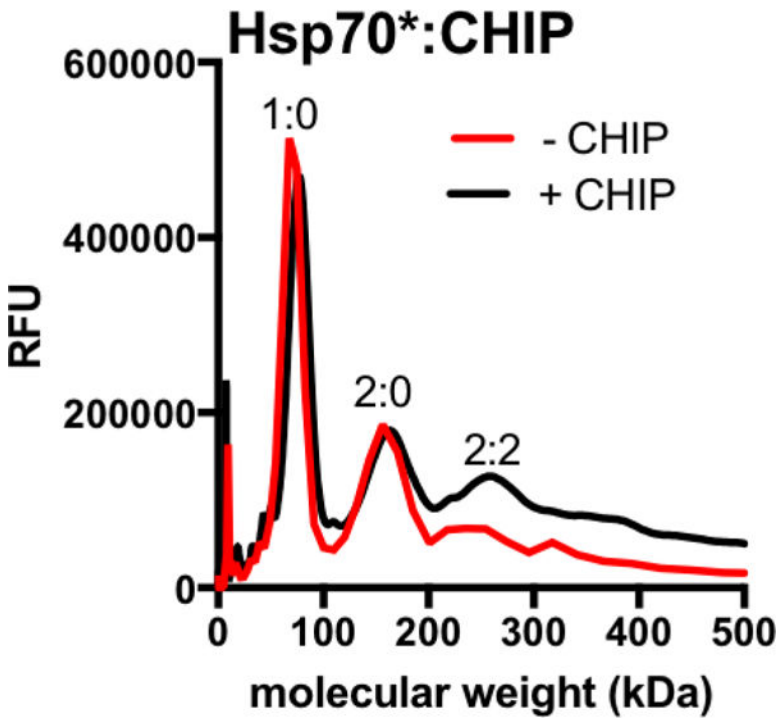

Figure 7.

Electropherograms of multimeric complexes $Hsp70_{apo}$:CHIP. Hsp70* concentration is 1 μM (both traces) and CHIP concentration is 4 μM (black track). Molecular weight was calculated based on size standards.