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A high throughput dimer screening assay for monoclonal antibodies using chemical cross-linking and microchip electrophoresis

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ABSTRACT

A high throughput screening assay was developed to determine the total dimer level in antibody samples. This method utilizes high speed microchip electrophoresis separation following chemical cross-linking. Upon reacting with homobifunctional *N*-hydroxysuccinimide-esters (NHS-esters), covalent linkages can be established between the primary amines of two neighboring antibody molecules. The reaction conditions are optimized to achieve quantitative cross-linking of only physically associated monomers within an antibody dimer. The resulting cross-linked dimers, originating from either covalent or non-covalent antibody dimers, can then be separated from monomers by SDS electrophoresis. A commercial microchip electrophoresis instrument is used for high speed separation, allowing each sample to be analyzed in about 1 min. This approach was applied to crude mammalian cell culture samples. Using a 96-well gel filtration spin column format, interfering species in the cell culture media were efficiently removed from the samples. This method is well suited to the purpose of high throughput antibody dimer quantitation during cell culture expression, including clone selection and cell culture development. The total dimer content, both covalent and non-covalent, can be determined for hundreds of crude samples in a few hours. The effects of different cross-linking conditions on the determined dimer levels, as well as of different antibody p*I* values, are discussed.

1. Introduction

Monoclonal antibodies (Mabs) are an important class of protein therapeutics used for a wide variety of disease indications [1]. For drug production, human or humanized versions of these proteins are typically expressed in Chinese hamster ovary (CHO) or other mammalian tissue culture cells in order to generate the disulfide structures and glycosylation patterns found in endogenous human antibodies. Purification processes are developed to purify the secreted antibody away from host cell proteins, DNA, and any potential viruses and to reduce product heterogeneity [2]. Of the many types of product heterogeneity, product aggregation has been a particular area of focus during process development and production [3].

Protein aggregation, or protein self-association, encompasses a large assortment of forms, which vary in both size and linkage type. These forms range in size from dimers to visible particles containing thousands of molecules which can associate either through covalent or non-covalent bonds. Larger aggregates can be more easily removed during a purification process, either by filtration or other means. Dimers and other small soluble associations may be more difficult to remove because their properties are more similar to those of the antibody monomer. While chromatographic techniques to remove dimers and other oligomers are available, it is desirable to reduce their levels during protein production. The level of dimer in expressed protein can vary in different clones or cell culture conditions. Therefore measurement of dimer is necessary during cell culture development. However, the number of experiments required for screening and optimization of cell culture conditions may greatly exceed the capacity of a bioanalytical laboratory using traditional analytical methods. With the recent development of high throughput bioreactors, such as the SimCell^{TM} bioreactor (BioProcessors Corp., Woburn, MA), product quality samples can number in the thousands from a single experiment. Measuring protein aggregation in such large numbers of crude cell culture samples poses a significant demand that is difficult to meet using conventional methods.

Abbreviations: NHS, *N*-hydroxysuccinimide; Mab, monoclonal antibody; CHO, Chinese hamster ovary; SEC, size exclusion chromatography; CE-SDS, capillary electrophoresis-sodium dodecyl sulfate; DMSO, dimethylsulfoxide; EGS, ethylene glycol bis(succinimidylsuccinate); Bis(NHS)PEO_5, bis *N*-succinimidyl-(pentaethylene glycol) ester; DST, disuccinimidyl tartrate; DSS, disuccinimidyl suberate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Table 1
Antibodies analyzed in this study.

Antibody	Description	p*I*
Mab A	Human, IgG2	7.44
Mab B	Human, IgG1	8.66
Mab C	Human, IgG1	8.78
Mab D	Human, IgG2	8.83
Mab E	Human, IgG2	7.40

Size exclusion chromatography (SEC) is often used in bioanalytical labs to determine total soluble protein aggregate level. Both covalent and non-covalent aggregates can be separated from monomers on analytical SEC columns, since the elution buffer is chosen to preserve non-covalent associations. For crude cell culture samples, this technique typically requires sample purification, such as protein A affinity, to remove interferences from matrix proteins in cell culture. SEC analysis may require 30 min or more for each sample, with additional time for preparation. Another relatively new technique to measure soluble low order aggregates, electrospray differential mobility analysis, works on a time scale similar to SEC [4]. Covalent dimers can also be quantified by capillary electrophoresis-sodium dodecyl sulfate (CE-SDS). When performed in a microchip format, each sample analysis can be performed in less than 1 min [5]. However, non-covalent dimers cannot be detected by this technique as they dissociate into monomers during sample preparation in SDS buffer.

Alternatively, chemical cross-linking can be used to convert non-covalent aggregates into covalent aggregates. The total aggregate levels can then be determined by subsequent SDS electrophoresis. Chemical cross-linking has been widely used to study protein–protein interactions [6,7], protein conformation [8], or to immobilize proteins onto surfaces for subsequent studies [9,10]. Although chemical cross-linking has also been used to identify the presence of protein aggregates [11,12], quantitative cross-linking for the purpose of determining total protein aggregate levels is not commonly used.

We describe here an analytical strategy to determine the total dimer level in antibody samples using chemical cross-linking combined with microchip CE-SDS. The impact of different cross-linking conditions on the results, as well as the overall assay performance, is addressed. The application of this method to crude cell culture samples is also demonstrated.

2. Materials and methods

2.1. Materials

HT protein express kits, including chips and reagents, were purchased from Caliper Life Sciences (Mountain View, CA). All monoclonal antibodies tested were expressed in CHO cells and produced at Amgen, Inc. (Thousand Oaks, CA). Five antibodies studied in detail are listed in Table 1. Zeba™ 96-well desalt spin plate and the following cross-linkers were purchased from Pierce (Rockford, IL): ethylene glycol bis(succinimidylsuccinate) (EGS); bis *N*-succinimidyl-(pentaethylene glycol) ester (bis(NHS)PEO_5); disuccinimidyl tartrate (DST); disuccinimidyl suberate (DSS). Dimethylsulfoxide (DMSO) was HPLC-reagent grade.

2.2. SEC analysis

SEC analyses were performed using Agilent Series 1100 binary pump system with UV detection at 280 nm and a flow rate of 0.5 mL/min. For all antibodies except for Mab A, up to 300 μg of antibody was injected onto a TSK-Gel G3000SW_{XL} column (5 μm, 7.8 mm × 300 mm, Phenomenex). The mobile phase was 100 mM sodium phosphate, 250 mM sodium chloride, pH 6.8. For SEC analysis of Mab A samples, 10–100 μg of antibody was injected onto a Shodex Protein KW-803 column (5 μm, 8 mm × 300 mm, Phenomenex). The mobile phase was 50 mM sodium phosphate, 250 mM sodium chloride, pH 7.0. For molecular weight determination, a miniDawn Tristar Laser Photometer operating at 690 nm (Wyatt) and an Optilab rEX Refractometer (Wyatt) were used in series with the UV detector. The molecular weight calculation used a d*n*/d*c* of 0.185 RIU-mL/g and a refractive index of 1.330.

2.3. Antibody cross-linking

On a 96-well plate, 35 μL antibody sample at approximately 0.4 mg/mL was mixed with 35 μL reaction buffer (20 mM sodium carbonate, pH 10.5) and 3.5 μL cross-linking reagent (80 mM EGS or bis(NHS)PEO_5 in DMSO). The resulting solution was then incubated at room temperature for 30 min. Other cross-linking conditions explored are described individually in the text. For cell culture samples, a desalting step was applied prior to the cross-linking reaction. The Zeba 96-well desalt spin plate was first centrifuged at approximately 1000 × *g* for 3 min to remove the storage solution. Cell culture samples (50 μL) at 0.7–1.3 mg/mL were then applied to each well, followed by centrifugation at about 1000 × *g* for 3 min. The eluate was then mixed with 75 μL water. A 35 μL aliquot of the resulting antibody solution (0.3–0.5 mg/mL) was then transferred to a 96-well plate for subsequent cross-linking reaction.

2.4. Microchip CE-SDS

High throughput analyses of the antibody cross-linking mixtures were performed on the LabChip 90 instrument (Caliper Life Sciences). Details of the microchip electrophoresis using LabChip 90 have been described previously [5]. Prior to loading the sample plate onto the instrument, 35 μL HT protein express sample buffer was added to each well of antibody cross-linking reaction mixture, followed by incubation at 70 °C for 15 min. The instrument controlling parameters were modified to allow longer separation time, resulting in 56 s per injection.

3. Results and discussion

3.1. Optimization of cross-linking conditions

A chemical cross-linking strategy has been developed to quantitatively preserve dimers during denaturing capillary electrophoresis, while at the same time minimizing artifactual cross-linking of monomeric species. Antibody samples composed primarily of either monomer or dimer, or a mixture of both were used to optimize conditions. Testing cross-linking reaction conditions on dimer and monomer samples independently allowed us to monitor authentic dimer cross-linking efficiency and artifactual dimer for each condition. The compositions of the initial developmental test samples are shown in Fig. 1. A monoclonal antibody (Mab A) preparative purification column fraction (Mab A-II) containing a mixture of dimer and monomer (Fig. 1d) was further enriched for dimer (Mab A-III, Fig. 1g) using size exclusion chromatography under native conditions. The purified Mab A (Mab A-I) composition, containing little dimer, is shown in Fig. 1a. The identities of the monomer and dimer peaks were confirmed by on-line multiangle light scattering. Masses of the monomer and dimer peaks in the SEC chromatograms were determined to be 1.4×10^5 g/mol and $2.7–3.1 \times 10^5$ g/mol, respectively, consistent with the assigned identities. For the purposes of these experiments, SEC chromatography was used to determine the levels of dimer and monomer in each sample. Dimer surviving the SEC chromatography is defined as physically associated monomers.

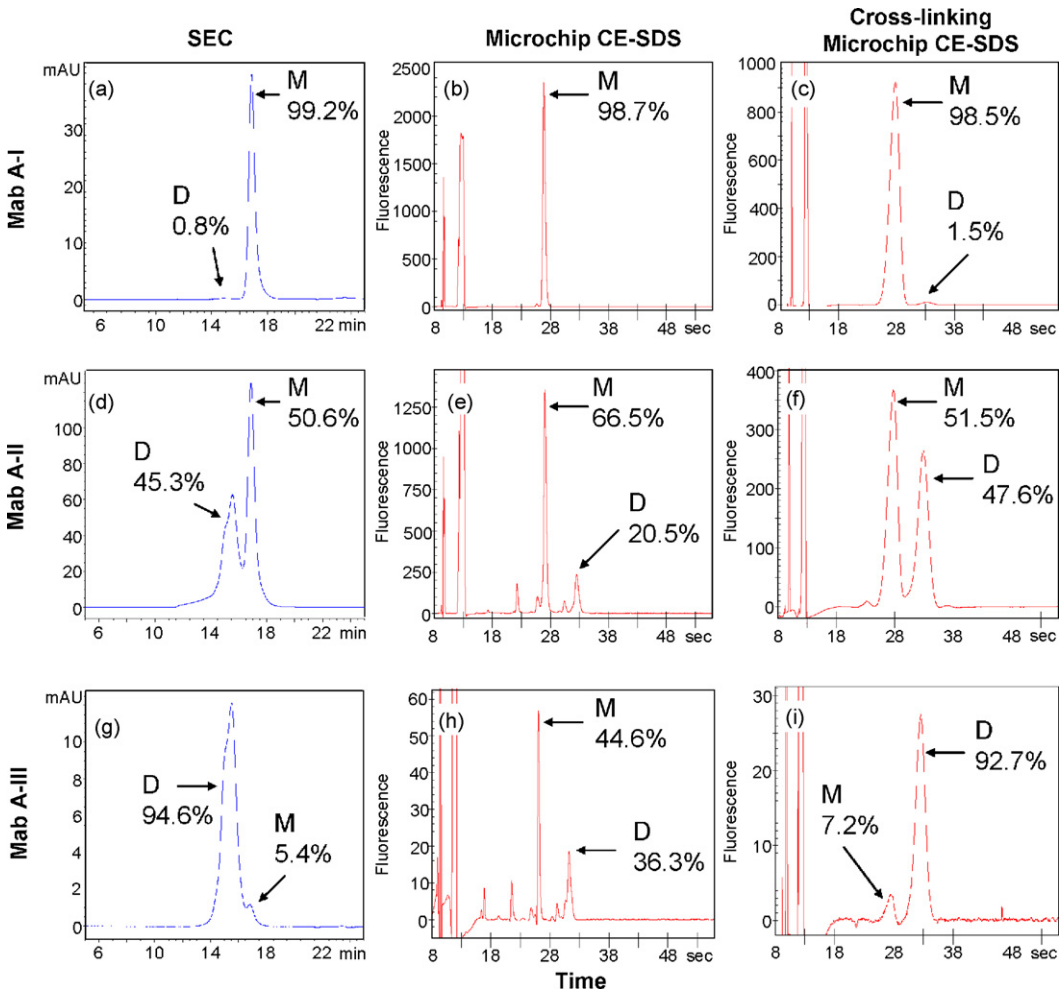


Fig. 1. SEC with online UV absorbance and light scattering detections (a, d, g), microchip CE-SDS (b, e, h), and cross-linking/microchip CE-SDS (c, f, i) analysis of Mab A-I, II, and III samples. Each SEC analysis takes 35 min. The buffer peak eluting at approximately 24 min was not included. The cross-linking reactions were performed as described in Section 2 using bis(NHS)PEO_5 as the cross-linker. M, monomer; D, dimer.

When these same samples (Mab A-I, Mab A-II, and Mab A-III) were analyzed by microchip based CE-SDS under denaturing but non-reducing conditions, the dimer content for all three samples was considerably reduced (Fig. 1b, e, h). For example, Mab A-III dimer content by CE-SDS is about 36% of the total material as compared to approximately 95% by SEC, indicating that approximately 40% of the Mab dimer is covalently linked. After cross-linking with the homobifunctional cross-linker bis(NHS)PEO_5, the levels of dimer on non-reducing microchip CE-SDS (Fig. 1c, f, i) more closely match those determined by SEC. Covalent bonds were generated between Mab A within dimers in the cross-linking reactions, preventing their dissociation upon heating in SDS. Noticeable broadening of both monomer and dimer peaks in microchip CE-SDS occurs upon treatment with the cross-linker, likely due to a wider molecular size distribution caused by heterogeneous cross-linker decoration of the antibody molecules.

As mentioned above, quantitative determination of the total dimer level in the antibody samples (comprised of both covalent and non-covalent forms) requires efficient cross-linking of authentic dimers without artifactually cross-linking the antibody monomers. To monitor both phenomena separately, monomer (Mab A-I) and dimer (Mab A-III) samples were utilized to screen different cross-linking conditions. In such screening experiments, the concentration of the dimer control (Mab A-III) was kept at 10% of the monomer control (Mab A-I), to optimize conditions around a theoretical sample containing 10% dimer. Products of the reactions were analyzed by non-reducing microchip CE-SDS, and compared to expected SEC results. Under optimum conditions, the Mab A-III dimer percentage is expected to be approximately 95%, whereas that of Mab A-I should be approximately 1%.

There are a number of factors that can impact the efficiency of protein chemical cross-linking reactions: cross-linker specificity, spacer arm length, and reaction conditions such as reaction time, temperature, pH, and concentrations of protein and cross-linker.

3.1.1. Selection of cross-linker

A variety of chemical cross-linking reagents are commercially available with different chemical selectivities. Cross-linkers of the NHS-ester family are chosen in this application because they react with primary amine groups at high efficiency and yield stable coupling products [13]. The large number of primary amine functional groups found on each antibody molecule, either lysine side chains or *N*-terminal amine, increases the probability that an amine specific cross-linker will be able to react with groups on each monomer within a dimer. A few NHS-ester chemical cross-linkers with different spacer arm lengths were tested. Under some conditions promoting dimer cross-linking in the Mab A-III sample (dimer percentage 90–95%), significant levels of artifactual dimers were also found in the Mab A-I sample. Cross-linkers with shorter spacer arms, such as DST (6.4 Å) and DSS (11.4 Å), were more prone to this effect than ones containing longer spacer arms, such as EGS (16.1 Å) or bis(NHS)PEO_5 (21.7 Å). Results obtained using DSS and

Table 2
%Dimer of Mab A-I determined at different protein concentrations and with different cross-linkers[a].

Protein concentration	4.0 mg/mL	2.0 mg/mL	0.5 mg/mL	0.25 mg/mL	0.125 mg/mL
DSS	19.7%	15.5%	8.5%	4.6%	3.2%
EGS	ND[b]	ND[b]	3.9%	2.5%	1.6%

[a] The cross-linking reactions were conducted at pH 7.0 in HEPES buffer with a cross-linker concentration of 0.5 mM. All concentrations indicated correspond to the final concentrations in the reaction mixture.
[b] ND, not determined.

EGS are compared in Table 2. With the same protein concentrations, dimer percentages measured in Mab A-I DSS reactions were twice that of EGS reactions. The two longest cross-linkers tested, EGS and bis(NHS)PEO_5, were considered interchangeable, since the differences between the two cross-linkers were small under a variety of conditions tested. This is demonstrated in Fig. 2a, where the dimer percentages for Mab A-I, II, and III measured under different cross-linker concentrations are shown to be very similar between EGS and bis(NHS)PEO_5.

3.1.2. Effect of antibody concentration

Antibody concentration also plays a critical role in the cross-linking reaction. As can be seen in Table 2, the amount of artifactual dimer increases with higher protein concentrations. At antibody concentration of 0.5 mg/mL or lower, the percentage of artifactual dimer decreased to less than 4% when EGS or bis(NHS)PEO_5 was used (see Table 2). In addition, very similar results were obtained when the antibody concentration fell between 0.125 and 0.25 mg/mL, as illustrated in Fig. 2b. A final antibody concentration of 0.2 mg/mL in the cross-linking reaction mixture was thus chosen. It can also be concluded from these results that antibody samples with concentration difference of up to two fold can be treated identically in this method, allowing convenient sample preparation in high throughput applications.

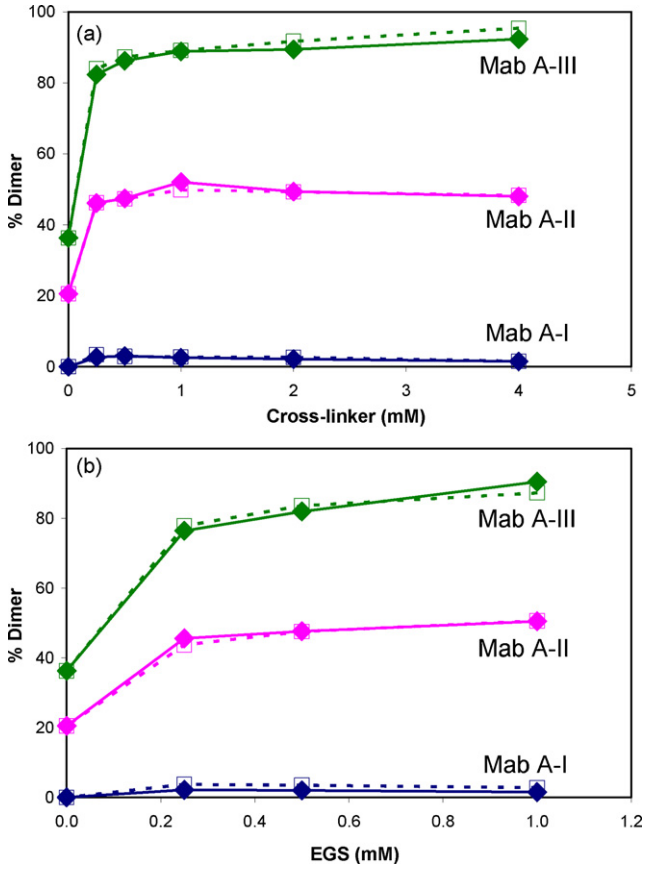


Fig. 2. (a) Effect of EGS and bis(NHS)PEO_5 cross-linker concentrations on %dimer of Mab A samples. Open square and dashed line, EGS; filled diamond and solid line, bis(NHS)PEO_5. The protein concentrations in the cross-linking reaction mixtures are 0.2, 0.2, and 0.02 mg/mL for Mab A-I, II, and III, respectively. (b) Comparisons of %dimer for Mab A samples at different EGS concentrations. Open square and dashed line, Mab A-I and II at 0.25 mg/mL and Mab A-III at 0.025 mg/mL. Filled diamond and solid line, Mab A-I and II at 0.125 mg/mL and Mab A-III at 0.0125 mg/mL. All reactions were conducted at room temperature for 30 min and at pH 7.0 in 10 mM HEPES.

3.1.3. Effect of cross-linker concentration

The cross-linker concentration was optimized. Results with EGS and bis(NHS)PEO_5 are shown in Fig. 2a. EGS or bis(NHS)PEO_5 concentrations higher than 1 mM provided the best dimer recovery. Mab A-II and III dimer levels reached plateaus of 45–50% and 90–95% dimer, respectively, with all the tested cross-linker concentrations exceeding 1 mM. Artifactual dimer appeared to reach a maximum at intermediate cross-linker concentrations. Although a small effect, the percentage of artifactual dimer observed for Mab A-I reached maximum of 3.0% at 0.5 mM bis(NHS)PEO_5, but decreased at higher cross-linker concentrations (1.5% at 4 mM bis(NHS)PEO_5). A possible explanation for the latter observation is that at high cross-linker and low protein concentrations the primary amines on the antibody monomer were quickly occupied by cross-linkers before two monomers come across with each other, thus preventing artifactual cross-linking of two monomer molecules. A cross-linker concentration of 4 mM was selected as the final reaction condition. Cross-linker concentrations higher than 4 mM were deemed less desirable due to excessive peak broadening, as discussed above.

3.1.4. Effect of antibody pI and reaction pH

The initial cross-linking conditions established with Mab A did not work as well with all antibodies tested. Several antibodies generated unacceptably high artifactual dimer levels. Interestingly, a correlation between dimer artifact and the protein's isoelectric point (p*I*) was found. Fig. 3a shows 14 different human or humanized monoclonal antibodies, both of IgG1 and IgG2 classes, ordered by their p*I* values (6.8–8.8). After treatment with 4 mM EGS at pH 7.0, a wide range of dimer levels was observed. Although all these antibodies were highly purified and contained less than 2% dimer by SEC analysis, some reactions generated dimer levels exceeding 30%. Molecules with higher p*I*'s appeared more prone to cross-linking artifact.

Cross-linking conditions were sought which could be applied universally to monoclonal antibodies. Reactions were tested over a wide range of pH values, from 5 to 11. As can be seen in Fig. 3b, artifactual dimers can be minimized at pH values below 5.0 or above 10.5. Since the cross-linking efficiency of authentic dimers also decreases significantly at lower pH values (Mab A-III shown in Fig. 3b) but not at higher pH values, pH 10.5 was selected as the pH for the universal antibody cross-linking reaction.

3.1.5. Reaction time and temperature

The cross-linking reaction time was evaluated at 10, 30, and 120 min. Although 10 min was sufficient under certain conditions, 30 min was selected to improve the reaction robustness with respect to time. For ease of operation, room temperature was

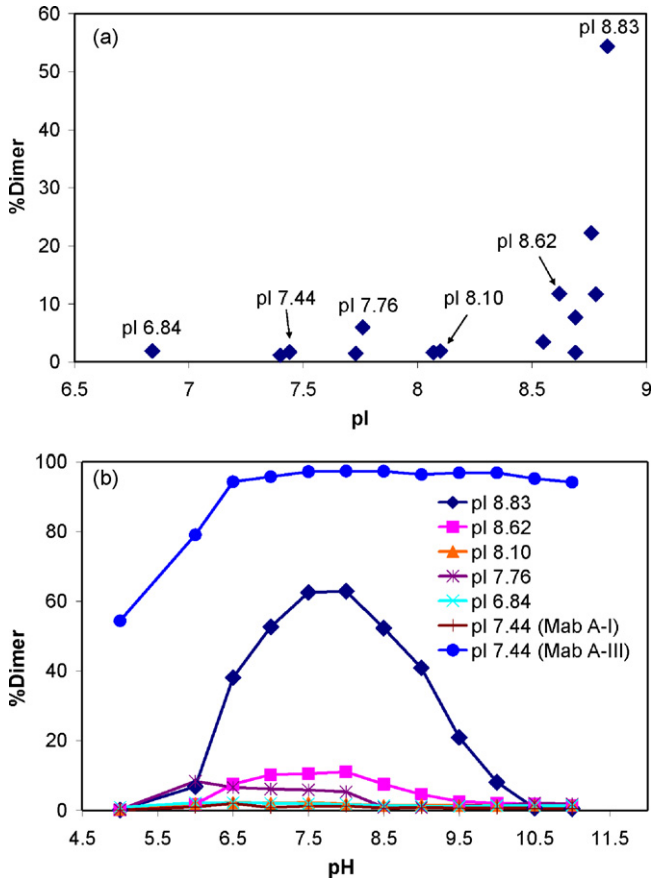


Fig. 3. The effect of antibody p*I* values (a) and reaction pH (b) on %dimer determined. All cross-linking reactions were conducted as described in Section 2 using EGS except that HEPES buffer at pH 7.0 was used in (a), and the following buffer systems were used in (b): sodium acetate, pH 5.0–6.0; sodium phosphate, pH 6.5; HEPES, pH 7.0–8.0; sodium borate, pH 8.5–10.0; sodium carbonate, pH 10.5–11.0. Note that MAb A-III sample is mainly dimer (as seen in Fig. 1g), so the dimer represents the efficiency of the reaction relative to reaction pH, not artifactual cross-linking.

selected as the reaction temperature without further development. The final optimized cross-linking conditions are described in Section 2.

3.2. Assay performance

3.2.1. Detection of aggregate size

Antibody samples also can contain aggregated species larger than dimer. For example, Fig. 4a shows a SEC chromatogram of a monoclonal antibody, Mab B, enriched in such higher order aggregates, collectively termed oligomers. This sample contained 3.8% dimer as well as 9.8% oligomers. The Mab B sample was analyzed by the cross-linking and microchip CE-SDS approach with the result shown in Fig. 4b. Similar dimer levels were measured by the native SEC and cross-linking/microchip CE-SDS methods. However, the oligomers observed in SEC were not detected by the cross-linking/microchip CE-SDS approach. Since the oligomer peak eluted at the SEC void volume, these species could be a mixture of species heterogeneous in molecular size, though no further work was performed to characterize these forms. The oligomers might not be detected by cross-linking/microchip CE-SDS because they are too large to migrate within the detection time window utilized here, or because each oligomeric form's abundance is below the detection limit of the microchip analysis. It is also possible that cross-linking of oligomers larger than dimer is much less efficient under the cross-linking conditions utilized here. Regardless of the

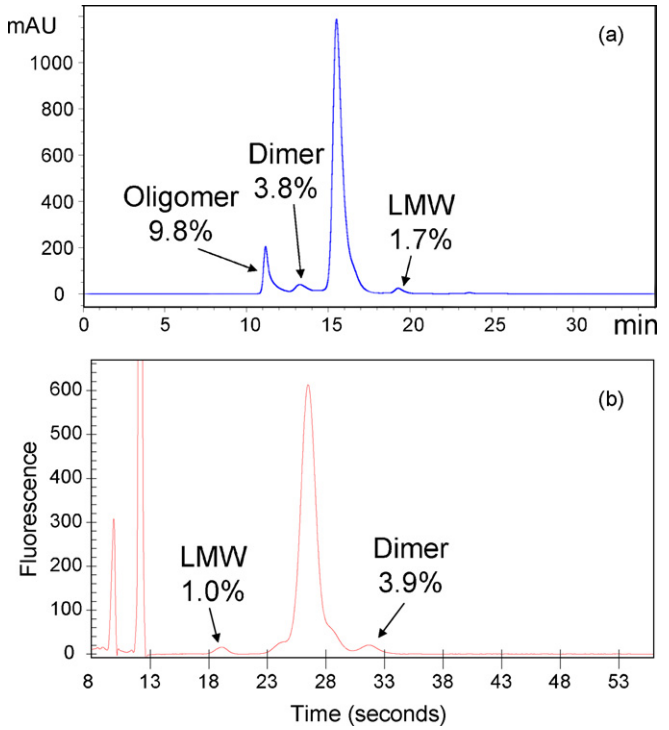


Fig. 4. Analysis of an aggregate enriched Mab B sample by SEC (a) and cross-linking/microchip CE-SDS (b). LMW, low molecular weight species.

mechanism, the cross-linking/microchip CE-SDS assay measures dimeric antibody species but not higher order oligomers.

3.2.2. Linearity

To demonstrate the linearity of the method, three antibodies (Mab C, Mab D, and Mab E), varying in dimer content were analyzed. Mab C is a fully human IgG1 and Mabs D and E are human IgG2s. For each antibody, a sample containing primarily monomer was blended with a dimer enriched sample at different ratios to generate a series of samples differing in dimer content. The measured dimer content using the cross-linking/microchip CE-SDS approach for these samples was then compared with the predicted SEC results. Correlation plots for these results are given in Fig. 5. Good linearity was obtained for the three antibody blending studies, all with the square of the correlation coefficients exceeding 0.99.

3.2.3. Limit of detection (LOD)

The limit of detection for dimer percentage at the nominal concentration of the original antibody sample prior to the cross-linking reaction (0.4 mg/mL) was calculated using two approaches. In the first approach, the LOD was calculated based on the dimer percentage linear plot (Fig. 5c) using the equation LOD = $3.3 \times \sigma$/slope, where σ is the standard deviation of either the *Y*-intercept or the residuals of the regression line, whichever is larger. The LOD using the linearity plot is 0.5% dimer, or 2 μg/mL at the nominal load. In the other approach, the LOD was calculated assuming a practical limit is at a signal to noise ration ratio (S/N) of 3–1. The LOD was estimated to be 0.2%, or 0.8 μg/mL, based on the peak height of the protein peak and the peak-to-peak baseline noise. The LOD determined from both approaches are comparable to antibodies analyzed by non-reducing microchip CE-SDS without prior cross-linking (1 μg/mL) [5]. It is worth pointing out that the majority of the overall assay variability might come from the cross-linking reaction rather than the microchip analysis.

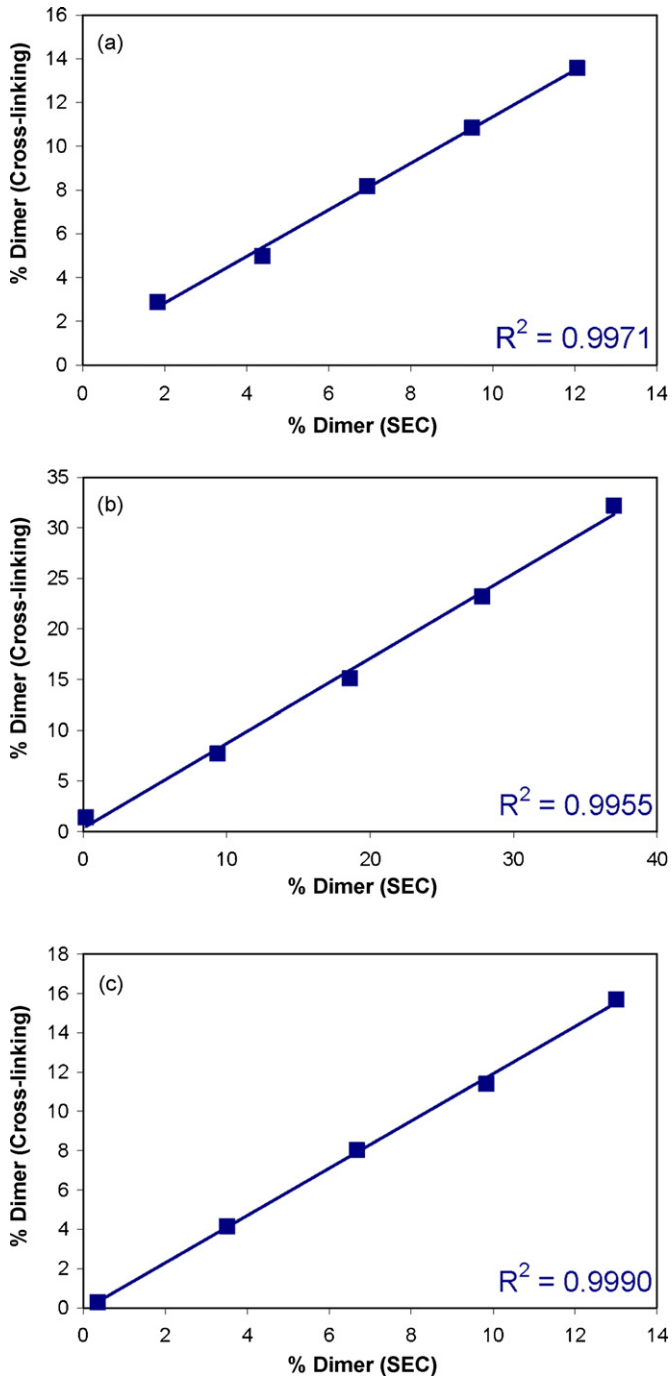


Fig. 5. Linearity of %dimer for Mab C (a), Mab D (b), and Mab E (c), determined by cross-linking/microchip CE-SDS.

3.2.4. Intermediate precision

The Mab A-II sample (SEC percentage dimer = 45.3%) was analyzed five separate times by two analysts on five different days using two instruments and separate microchips and reagents. Each experiment included the cross-linking reaction and microchip separation. An average cross-linking/microchip CE-SDS dimer value of 46.3% was obtained, with a standard deviation of 1.0%, generating a relative standard deviation (RSD) of 2.2%.

3.3. Analysis of cell culture samples

During process development, a large number of experiments are often performed to select high expressing clones and optimize cell culture conditions. If product attributes are monitored at this stage the number of samples generated can strain the capacities of the bioanalytical laboratory. However, since aggregate levels can be influenced by cell culture conditions, monitoring at this stage will help reduce aggregate levels and the impact on the purification process. Traditionally, monitoring antibody aggregate levels in crude cell culture samples required partial purification of the antibody, such as with protein A affinity, prior to aggregate analysis. For a SEC analysis, 30 min or more are required for each analysis. Thus, this approach is not well suited for high throughput cell culture development studies. The cross-linking/microchip CE-SDS approach could greatly increase throughput, especially for large numbers of samples, where the impact of the cross-linking reactions on throughput would be minimized by parallel sample preparations.

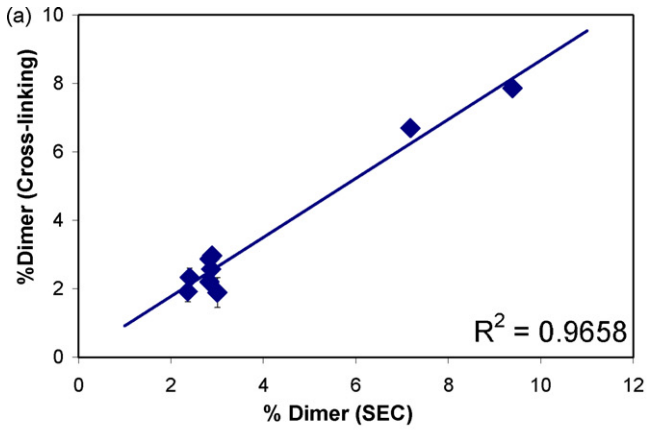
Table 3
Effect of cell culture media and desalting on %dimer determined by cross-linking/microchip CE-SDS. SEC results are also included for comparison.

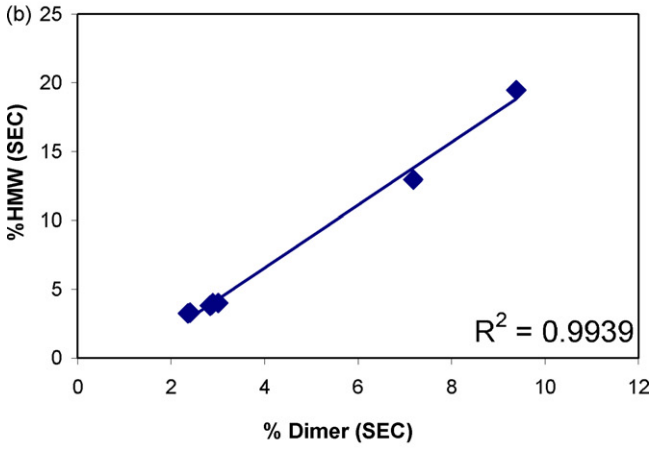
	SEC	Before spiking	Spiked into cell culture media	Desalting after spiking
Mab A-I	0.8%	0.5%	0.7%	0.3%
Mab A-II	45.3%	46.3%	37.4%	44.4%
Mab A-III	94.6%	90.3%	74.2%	91.3%

The cross-linking/microchip CE-SDS dimer assay was applied to cell culture samples. To test directly on crude samples, media components that interfere with the cross-linking reaction may need to be removed or reduced. Amino acids and small peptides found in cell culture media at milimolar concentrations will also react with the NHS cross-linkers, and thereby reduce the antibody dimer cross-linking efficiency.

Spin desalting was evaluated as a preparation step to remove interfering media components. To determine the effectiveness of small molecule removal and antibody recovery, solutions containing either 0.4 mg/mL Mab A-I or 0.02 mg/mL tryptophan were applied to the desalting plate. The recoveries of both antibody and tryptophan were then calculated based on the UV absorbance at 280 nm. Recovery of the antibody was 93.0 ± 2.9%, based on the results of six replicates using two separate plates, while that of tryptophan was only 1.0 ± 0.3%. Further studies were then conducted to evaluate the effectiveness of this simple sample preparation step for subsequent cross-linking analysis. Mab A-I, II, and III samples were spiked into representative cell culture media followed by subsequent cross-linking/microchip CE-SDS analysis. The results are shown in Table 3. Interference from matrix was observed, as the dimer levels in cell culture media were lower than similar samples in other buffers. This interference could be effectively removed by spin desalting, with dimer values approaching those of the original samples. This clean up step requires approximately 10 additional minutes, but since several 96-well desalting plates can be prepared in parallel, the overall impact on high throughput analysis would be negligible.

This cross-linking/microchip CE-SDS with desalting preparation approach was tested on authentic Mab E cell culture samples. The dimer levels using the cross-linking/microchip CE-SDS method following the simple desalting step were compared with those determined by SEC following protein A purification (Fig. 6a). Good linear correlation was observed (Fig. 6a), with $R^2 > 0.96$. It is worth pointing out that the dimer content is an important quality attribute of antibody products. When compared to larger oligomers, dimer monitoring during clone selection and cell culture development may be more crucial, since dimers might be expected to be more difficult to remove than oligomers with conventional chromatographic purification. The size difference between dimers and monomers are relatively small, and antibody dimer fractions are often less separated from the main peak on large scale purifica-





(c)

Dimer microChip	RSD (%)	Dimer SEC
1.9	23	3.0
1.9	16	2.4
2.2	5.3	2.8
2.3	12	2.4
2.6	2.9	2.9
2.9	2.9	2.8
3.0	2.3	2.9
6.7	0.65	7.2
7.9	0.76	9.4

Fig. 6. (a) %Dimer of Mab E crude samples from different cell culture conditions were determined by cross-linking/microchip CE-SDS (*Y* axis) and compared with that by SEC after protein A affinity purification (*X* axis). Error bars represent ±one standard deviation for the set of replicates ($n = 3$), where each replicate is an independent experiment comprised of cross-linking plus separation steps. (b) A linear relationship between SEC %HMW (high molecular weight spices) and %dimer for the same set of Mab E crude samples is shown. (c). Table showing the data plotted in (a).

tion columns. Moreover, the dimer level, once determined, might be used as an indicator of the total aggregate level. This is demonstrated in Fig. 6b, where a good correlation between the dimer level and the level of total aggregates can be readily seen. Although the exact linear relationship observed in Fig. 6b may not apply to all clones and cell culture conditions, a qualitative correlation between the levels of dimer and total aggregates is possible. The method described here can thus be used in early screening processes to identify clones or cell culture conditions producing high dimer levels, providing critical information to improve the purity and yield of the final antibody product.

4. Conclusions

A high throughput assay has been developed to determine total antibody dimer level utilizing chemical cross-linking followed by microchip CE-SDS analysis. The cross-linking reaction conditions have been optimized to achieve quantitative cross-linking of authentic dimers while minimizing artifactual cross-linking of monomers. Antibodies with a wide p*I* range (6.8–8.8) have been tested by this method. This method can also be applied to crude cell culture samples. A simple desalting step utilizing a 96-well desalt spin plate is efficient in removing interfering species from cell culture media. Both sample clean-up and subsequent cross-linking reactions can be readily performed in parallel fashion using 96-well plates and multi-channel pipettes or liquid handlers. The prepared sample plates can then be directly loaded on to a commercial microchip CE-SDS instrument for fast analysis. This method demonstrated good linearity for both IgG1 and IgG2 molecules, with good intermediate precision (RSD = 2.2%) and a LOD of less than 0.5% (2 μg/mL). This approach allows for the analysis of hundreds of samples, including crude cell culture samples, within a few hours, and is well suited for the purpose of high throughput dimer screening.

Conflict of interest

We declare no conflict of interest.

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