SimpleSol – a Novel Protein Solubilizer for Capillary Isoelectric Focusing Analysis

Introduction

Imaged capillary isoelectric focusing (iCIEF) is a standard method to assess charge heterogeneity, one of the critical quality attributes used to monitor a therapeutic protein's quality and consistency during development and manufacturing. However, protein aggregation and/or precipitation can occur during isoelectric focusing (IEF), resulting in non-reproducible peak profiles. Urea is a protein solubilizer commonly used to mitigate this aggregation and precipitation. Although effective, urea solutions must be freshly prepared immediately prior to use, adding an additional step and extra time to the sample analysis. Additionally, urea-induced carbamylation and anodic gradient drift cause apparent pI values to shift. Denaturing effects above 4 M are also observed with the use of urea.

This technical note introduces SimpleSol, a novel and alternative reagent to urea to solubilize proteins for icIEF analysis on iCE and Maurice systems. SimpleSol is a ready-to-use reagent that is significantly more stable compared to urea, eliminating the need to prepare fresh urea every day. SimpleSol is also more stable compared to urea when pre-mixed with methylcellulose and is compatible with both absorbance and native fluorescence detection with Maurice icIEF. Finally, SimpleSol affords significantly reduced anodic gradient drift than urea, which translates to more stable pI values for observed protein peaks. In summary, SimpleSol is a valuable, easy-to-use addition to the toolbox for analytical laboratories that routinely use additives to minimize aggregation and precipitation during icIEF analysis.

Material and Methods

SAMPLES

Human IgG1 kappa was obtained from Protos Immunoresearch (catalog #523), human recombinant EPO from R&D Systems (catalog #286-EP), and alpha-amylase from Sigma-Aldrich (catalog #A4551). NISTmAb, a reference humanized IgG1 kappa monoclonal antibody, was purchased from Sigma-Aldrich (catalog #NIST8671). The therapeutic fusion protein was obtained from an industry collaborator.

CIEF ANALYSIS ON MAURICE

When developing a new method using SimpleSol, we recommend screening a final concentration of 20–40% SimpleSol in your prepared sample and adjusting as necessary. At concentrations higher than 50%, focusing time can increase significantly.

To use SimpleSol, first add the appropriate volume of the reagent to your cIEF Master Mix. Then mix your cIEF Master Mix with your diluted sample. For example, to prepare 200 µL of a 0.17 mg/mL sample solution in 4% pH 3–10 Pharmalyte with 20% SimpleSol, prepare the cIEF Master Mix as shown in **Table 1**. Then add 34 µL of 1.0 mg/mL protein sample to 166 µL of cIEF Master Mix. Please refer to the Maurice and Maurice C. or iCE3 Method Development Guides for more information.

All samples were prepared as described below using reagents, including the SimpleSol, obtained from

the ProteinSimple cIEF Method Development Kit (PN PS-MDK01-C). Prepared samples were vortexed and centrifuged at 13,000 g for 3 minutes before the top 160 µL of the centrifuged solution was transferred to a 96-well plate or sample vials. The plate or sample vials were centrifuged again and placed in Maurice for analysis using a ProteinSimple Maurice cIEF cartridge (PN PS-MC02-C). The data were analyzed with Compass for iCE software in Absorbance (0.005 second exposure) and Native Fluorescence (5, 10, 15, and 20 second exposures).

Human IgG kappa was prepared in 0.35% methylcellulose, 2.56% 8–10.5 Pharmalyte, 2.05% 3–10 Pharmalyte, Maurice pI markers 7.05 and 9.50, and 0.15 mg/mL IgGk kappa. Samples included either no additive or 30% SimpleSol. The protein was separated for 1 minute at 1500 V, followed by 10 minutes at 3000 V.

Humanized NIST reference mAb was prepared in 0.28% methylcellulose, 0.60% 5–8 Pharmalyte, 3.40% 8–10.5 Pharmalyte, 4 mM iminodiacetic acid (IDA), 5 mM arginine, Maurice pI markers 4.05 and 9.99, and 0.20 mg/mL NISTmAb. Samples included either 3 M urea or 30% SimpleSol. The protein was separated for 0.5 minute at 1500 V, followed by 12 minutes at 3000 V.

Human recombinant EPO was prepared in 0.35% methylcellulose, 40% SimpleSol, 3% 2.5–5 Pharmalyte, 1% 5–8 Pharmalyte, 10 mM iminodiacetic acid (IDA), Maurice pI markers 3.38 and 5.85, and 0.3 mg/mL EPO. Samples included either no additive or 40% SimpleSol. The protein was separated for 1 minute at 1500 V, followed by 12 minutes at 3000 V.

The therapeutic fusion protein was prepared in 0.35% methylcellulose, 1% Servalyte mix (3:3:4 ratio of 3–5, 5–8, and 2–9 Servalytes), 50% SimpleSol, 4 mM arginine, 5.6 mM iminodiacetic acid (IDA), Maurice pI markers 4.05 and 9.50, and 0.45 mg/mL of protein. Samples included either no additive, 4 M urea, or 20% or 25% SimpleSol. The protein was separated for 1 minute at 1500 V, followed by 12 minutes at 3000 V.

Alpha-amylase was prepared in 0.28% methylcellulose, 4% 3–10 Pharmalyte, 4 mM iminodiacetic acid (IDA), 5 mM arginine, Maurice pI markers 3.38 and 10.17, and 0.15 mg/mL alpha-amylase. Samples included either 4 M urea or 30% SimpleSol. The protein was separated for 0.5 minute at 1500 V, followed by 6 minutes at 3000 V.

REAGENT	VOLUME
DI water	40 µL
1% Methylcellulose	70 µL
SimpleSol	40 µL
Phamalyte pH 3–10	8 µL
500 mM arginine (if necessary)	4 µL
High pI marker	2 µL
Low pI marker	2 µL
Total volume	166 µL

TABLE 1. cIEF Master Mix for a sample that contains a final concentration of 20% SimpleSol after sample addition.

A Maurice pI Marker Mix was also used to monitor the effects SimpleSol had on the pH gradient. This mix was prepared in 0.35% methylcellulose, 4% 3–10 Pharmalyte, 10 mM arginine, 10 mM iminodiacetic acid (IDA), and 2 µL each of the Maurice pI markers 3.38, 4.05, 5.85, 6.14, 7.05, 8.40, 9.50, 9.99, and 10.17. Samples either included no additive, SimpleSol (14, 20, 24, or 30%) or urea (1, 2, 3, or 4 M). The marker mix was separated for 1 minute at 1500 V, followed by 5 minutes at 3000 V.

SimpleSol is an Effective Solubilizing Agent

Three molecules known to require a solubilization agent for IEF were first analyzed using Maurice icIEF with and without SimpleSol. As expected, a significant amount of precipitation was observed with the therapeutic protein and a high degree of protein aggregation was observed with the recombinant EPO and Human IgG kappa (**Figure 1**). The addition of 20–50% SimpleSol to the prepared sample mitigates these issues, removing peaks in the profile indicative of protein aggregation and precipitation from the data. Injections comparing blank samples where no proteins were included in the sample mix demonstrate that SimpleSol does not affect the background absorbance (data not shown).

SimpleSol effectively solubilized the proteins analyzed, which translates to reproducible data across multiple injections. Quantitative analysis of 5 replicate injections of EPO run with 40% SimpleSol show great reproducibility in the apparent peak pI reported and the percent peak area (**Tables 2 and 3**). %RSDs for percent peak area were

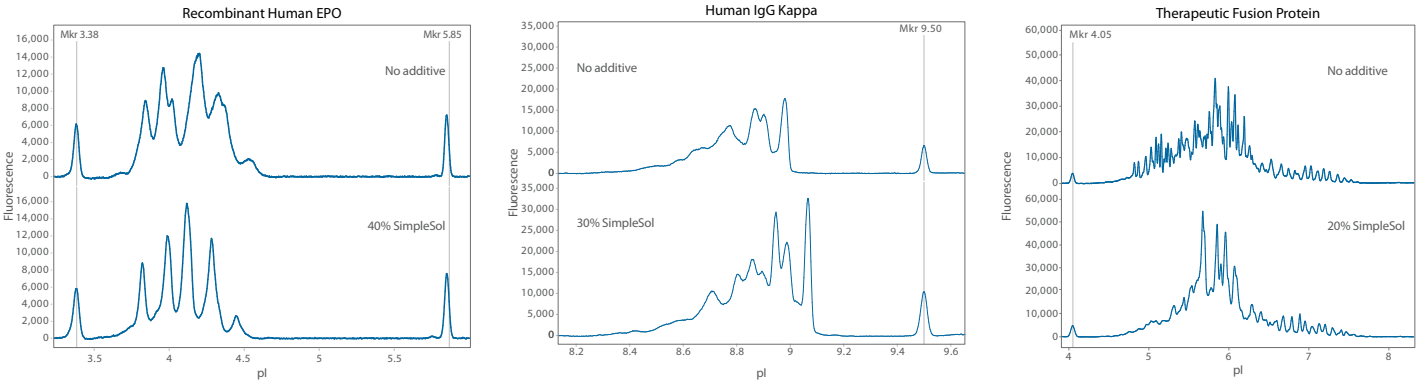

FIGURE 1. Comparison of recombinant human EPO, human IgG kappa, and a therapeutic fusion protein run on Maurice using native fluorescence detection with or without SimpleSol. The observed protein aggregation and precipitation observed when the proteins were analyzed without a solubilizing agent is not observed with the addition of SimpleSol.

all under 3% and reported pI value %RSDs were all below 0.05% for all five peaks.

Comparison of SimpleSol with Urea

Three molecules, a therapeutic fusion protein, alpha-amylase, and the NISTmAb, were then analyzed using either urea or SimpleSol on Maurice using absorbance and native fluorescence detection (**Figure 2**). The data indicated that 25% of SimpleSol was sufficient to remove all signs of protein aggregation in the therapeutic fusion protein that 4 M urea could not, resulting in better reproducibility when quadruplicate injections were overlayed. 30% SimpleSol also dramatically reduced the precipitation seen when alpha-amylase was analyzed with 4 M urea. There was also an acidic shift observed with the alpha-amylase in the sample analyzed with urea compared to SimpleSol, and the resulting peak profile with SimpleSol was closer to the theoretical pI value for alpha-amylase.

Overlaying triplicate injections of the NISTmAb prepared with 3 M urea or 30% SimpleSol demonstrated both sufficiently solubilized the NISTmAb, but that there was better resolution between the main peak and the acidic peak observed with the sample prepared with SimpleSol.

SimpleSol has Minimal Impact on the pH Gradient and Apparent pI

One of the drawbacks of using urea as a protein solubilizer is that urea can cause anodic gradient drift, which will cause apparent pI values to change. To assess the effect of SimpleSol on the pH gradient, a mixture of Maurice pI markers was prepared with 1–4 M urea or 14–30% SimpleSol (**Figure 3, left**) and run on Maurice. A no additive control was also run as a reference. Urea induced a larger shift in pixel position for the acidic pI marker 3.38, as there was an average anodic shift of 106 pixels in samples with 4 M urea compared to the no additive

PEAK	AVERAGE pI	RSD
Peak 1	4.46	0.02%
Peak 2	4.29	0.04%
Peak 3	4.11	0.01%
Peak 4	3.93	0.02%
Peak 5	3.79	0.03%

TABLE 2. Quantitative analysis of the reported peak pI for triplicate injections is reproducible. The recombinant EPO was analyzed with 40% SimpleSol using Maurice icIEF native fluorescence. The data were highly reproducible, with %RSDs all below 0.05% for all five peaks.

PEAK	AVERAGE % PEAK AREA	RSD
Peak 1	5.57	1.50%
Peak 2	23.70	0.58%
Peak 3	30.29	1.07%
Peak 4	23.73	1.43%
Peak 5	13.16	2.83%

TABLE 3. Quantitative analysis of the % peak area for triplicate injections is reproducible. The recombinant EPO was analyzed with 40% SimpleSol using Maurice icIEF native fluorescence. The data were highly reproducible, with %RSDs all below 3% for all five peaks.

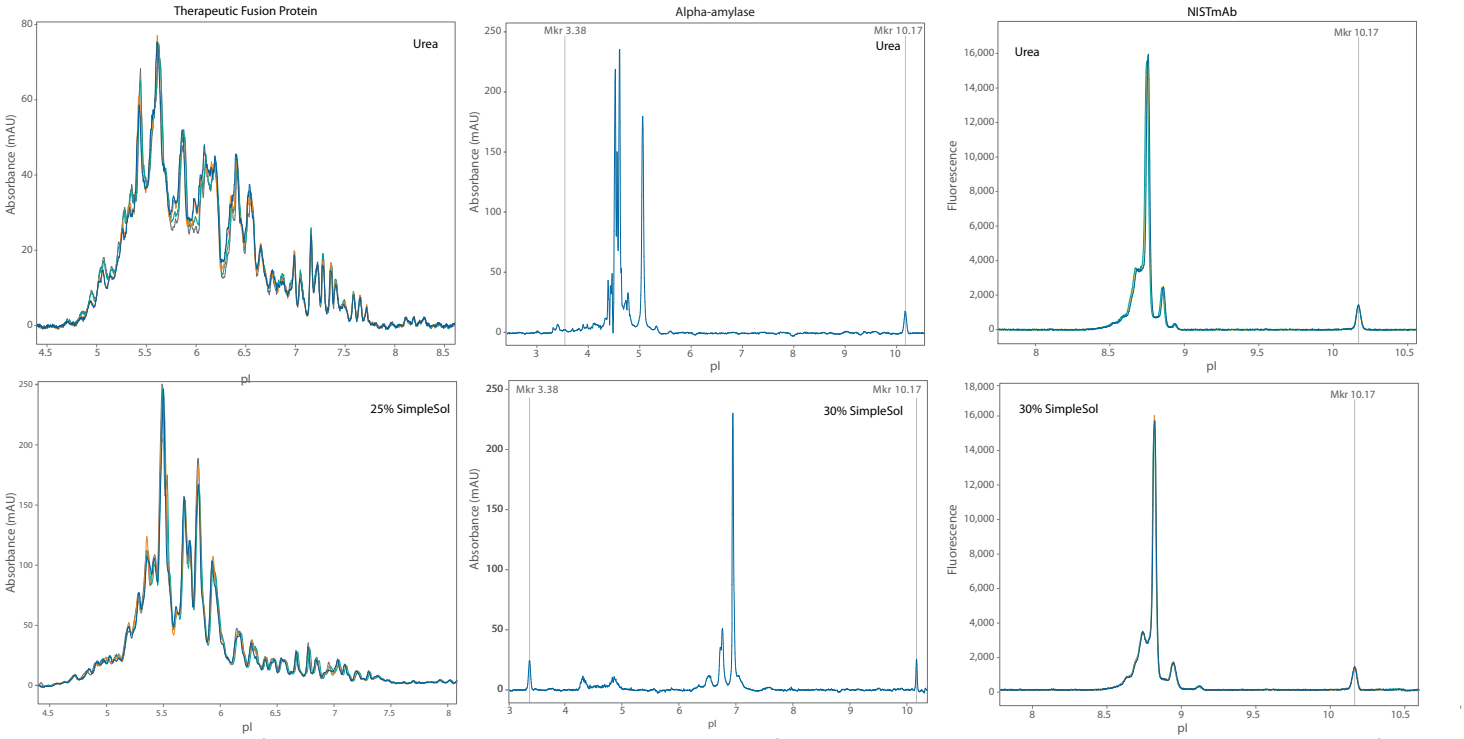

FIGURE 2. Comparison of urea and SimpleSol indicates SimpleSol can be used for samples where urea does not completely remove all signs of protein aggregation for the therapeutic fusion protein or precipitation for alpha-amylase. Increased resolution was also observed when SimpleSol was used instead of urea to analyze the NISTmAb.

control. In comparison, 30% SimpleSol only showed an average shift of 44 pixels (**Figure 3, right**). In general, all of the SimpleSol concentrations tested reported apparent pI values that were closer to the no additive control and exhibited smaller differences compared to the different concentrations of urea tested (**Figure 4**).

SimpleSol is More Stable Compared to Urea

Urea degrades quickly and must be prepared fresh every day to generate reproducible icIEF results between different batches on Maurice and iCE systems. To compare

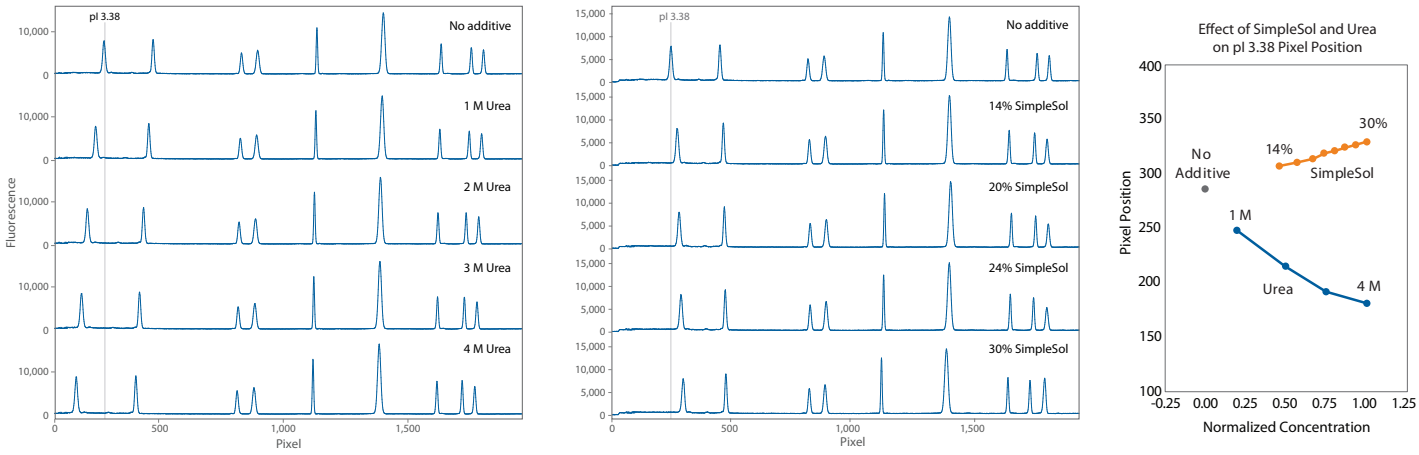

FIGURE 3. More acidic drift was observed with urea compared to SimpleSol. A more pronounced shift in the pixel position for Maurice pI standard 3.38 was observed in increasing amounts of urea compared to SimpleSol (left and middle). 4 M Urea induced an average anodic shift of 106 pixels compared to the no additive control (right). 30% SimpleSol only caused an average anodic shift of 44 pixels compared to the no additive control.

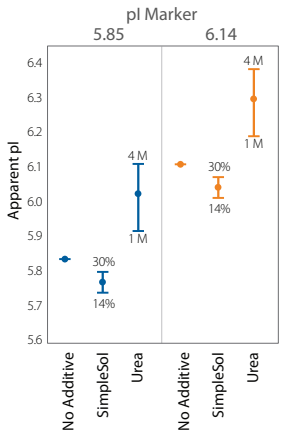

FIGURE 4. The acidic drift observed with the addition of solubilizing agents results in differences in the reported apparent pI. SimpleSol had less acidic drift, resulting in a smaller difference in the reported apparent pI for Maurice pI markers 5.85 and 6.14 compared to urea.

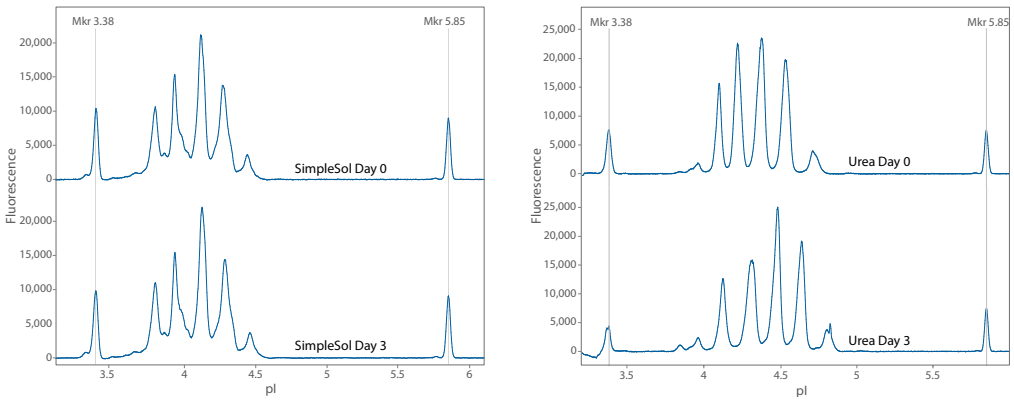

FIGURE 5. SimpleSol is more stable than urea for samples analyzed with icIEF. Human EPO analyzed with fresh and aged urea exhibited a change in peak profile, including shifts in the apparent pI values and percent peak area compositions, not observed with EPO analyzed with fresh and aged SimpleSol.

the stability of the two solubilizing agents, Human EPO was run with urea and SimpleSol that was prepared fresh (Day 0) or aged for 3 days at 4 °C (**Figure 5**). Differences between urea and SimpleSol in the apparent pI on day 0 for each of the peaks can be attributed to the different impact each additive has on the acidic portion of the pH gradient.

pI VALUE				
PEAK	UREA DAY 0	UREA DAY 3	SIMPLESOL DAY 0	SIMPLESOL DAY 3
Peak1	4.71	4.81	4.44	4.46
Peak2	4.53	4.64	4.27	4.29
Peak3	4.37	4.48	4.12	4.13
Peak4	4.22	4.30	3.93	3.93
Peak5	4.10	4.13	3.80	3.80

TABLE 4. Differences in apparent pI values were greater in human EPO analyzed with aged urea compared to aged SimpleSol. Urea reported pI differences of 0.03–0.11 pI units between fresh and aged urea while fresh and aged SimpleSol reported pI differences of just 0.00–0.02 pI units.

% PEAK AREA				
PEAK	UREA DAY 0	UREA DAY 3	SIMPLESOL DAY 0	SIMPLESOL DAY 3
Peak1	5.43	5.14	5.56	5.58
Peak2	24.52	22.67	25.13	25.17
Peak3	29.52	28.94	29.11	29.55
Peak4	24.30	24.68	23.61	23.28
Peak5	13.57	13.99	16.59	16.42

TABLE 5. SimpleSol is more stable than urea, resulting in percent peak compositions that are more consistent between day 0 (fresh reagent) and day 3 (aged reagent).

The EPO that was run with aged urea showed a change in the peak profile compared to the run with fresh urea, including a shift in the apparent pI values and the percent peak area compositions (**Tables 4 and 5**). In contrast, fresh and aged SimpleSol performed similarly, confirming that SimpleSol is more stable.

Conclusion

SimpleSol is a novel protein solubilizer that further simplifies the Maurice and iCE3 workflow by addressing the aggregation and precipitation issues often observed with icIEF analysis. It is compatible with icIEF analysis of proteins using both UV absorbance and native fluorescence detection on the Maurice system, and produces highly reproducible peak profiles as measured by peak percent area. SimpleSol also reports more consistent apparent pI values than urea because it has less of an impact on gradient formation. It is provided as a ready-to-use solution that is highly stable, and in some cases is a more effective solubilizer than urea or other additives. If needed, SimpleSol can be used in combination with urea. In summary, SimpleSol is a powerful addition to your cIEF method development toolkit that simplifies your workflow while still delivering reproducible results.

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