Evaluation of Laser Induced Native Fluorescence at 224 nm for Protein Analysis by SDS – Capillary Gel Electrophoresis

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Introduction

The CE-SDS-IgG Purity Assay is an industry-approved technology in the manufacture and quality control of therapeutic proteins. The current method with UV absorbance has a detection limit similar to coomassie blue SDS-PAGE and there has been interest to improve sensitivity to the level of silver stain SDS assay. One established approach to enhance sensitivity is to utilize laser-induced fluorescence (LIF) detection of fluorescently labeled proteins. This strategy has been shown to be robust but often depends on protein sequence which can be variable and thus contribute to concern regarding quantitation. In an effort to simplify the assay and allow for more quantifiable results, we are exploring techniques in which native fluorescent properties of proteins can be used as an alternative to labeling technology.

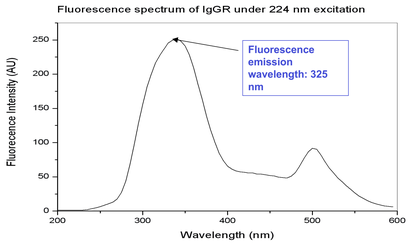
Most native fluorescence detection takes advantage of the intrinsic fluorescence of tyrosine, tryptophan and phenylalanine that contains aromatic rings using excitation laser of 266 nm or above. However this approach is problematic as the fluorescence response is amino acid sequence dependent, and therefore cause error to protein quantitation. In this study, we explored the feasibility of low wavelength UV laser for generic protein native fluorescence that is not protein sequence dependent. A prototype 224 nm laser induced native fluorescence (LINF) detector was adapted to the Beckman Coulter PA 800 Protein Characterization System. The quantitative results of IgG analysis using LINF will be compared with UV detection and analysis of high molecular weight IgG aggregates will also be discussed.

Issue with Protein Detection Based on Aromatic Amino Acid Residues

When UV absorption of 260 nm or above was utilized, protein absorption is mainly contributed by aromatic amino acid residues, causing error in quantitation due to sequence based response.

UV Absorbance	Ratio of HC/LC
220 nm	2.089
254 nm	2.381
265 nm	3.107
280 nm	3.144

Table 1. A reduced mouse IgG 1kappa (1 mg/ml) was analyzed using PDA detector with UV scan from 200nm to 300 nm. The ratio of HC/LC was then analyzed at different UV absorption wavelength.
Separation conditions: 30cm, 50mm ID uncoated capillary. Injection was 5kV for 20 seconds. Separation voltage was 15kV with 20psi at both ends of the capillary.



A prototype laser induced native fluorescence detector was externally adapted to PA 800 system

Accurate Quantitation of IgG Purity Using LINF Detection

The quantitative analysis results of LINF with excitation at 224 nm is consistent with PDA detector with absorbance detection at 220 nm.

	Integration	HC/LC	NG%
PDA Detector	Corrected area	2.07	11.20%
	Peak aera	2.61	11.00%
LINF Detector	Corrected area	2.13	10.37%
	Peak aera	2.69	10.08%

Table 2: Reduced mouse IgG 1kappa (1 mg/ml) were analyzed on LINF and PDA detector respectively. Separation condition: for PDA, separation was performed at 25°C in a 30cm, 50mm ID uncoated capillary at 15kV with 20psi pressure at both ends. Injection was 5kV for 20 s. For LINF, separation was performed at 25°C in a 100cm, 50mm ID uncoated capillary at 28 kV. Injection was 10kV for 30 s. LINF excitation was at 224nm and emission at 325nm.

Sensitivity Improvement of LINF Compared to UV

Sensitivity was improved compared to UV detection using prototype external LINF detector. The detection system will be further optimized to determine if it can achieve sensitivity levels of silver stain SDS assay

PDA Detector:
50 µm ID, 30 cm capillary, 20 cm to detection window

LINF Detector:
50 µm ID, 100 cm capillary, 85 cm to detection window

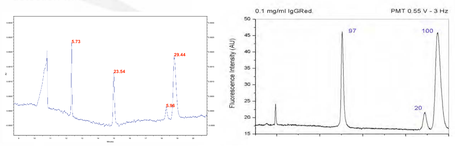


Figure 1. SDS-CGE separation of reduced IgG control standard (0.1 mg/ml) with LINF and PDA detection. Separation conditions are the same as described in Table 2. Numbers above the peak represent calculated signal to noise.

Linearity of LINF Detection

Fluorescence detection response is linear to IgG concentration through range of 0.01 mg/ml to 1 mg/ml

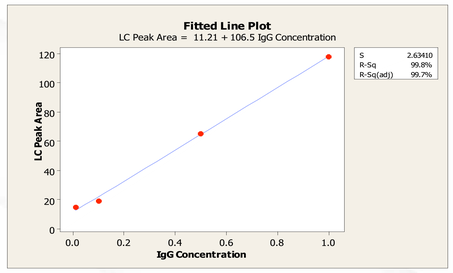


Figure 2. Linearity of LINF detector.
Reduced mouse IgG 1 Kappa (from 0.01 mg/ml to 1 mg/ml) was used as test sample. Separation was performed at 25°C in a 100 cm, 50mm ID uncoated capillary (85 cm to detection window). Injection was 10kV for 30 seconds. Separation voltage was 15kV. LINF excitation was at 224nm and emission at 325nm.

Separation of Non-reduced IgG Using SDS-CGE with LINF Detection

High sensitivity and stable baseline were obtained using the LINF detector, allowing analysis of high molecular weight IgG aggregates.

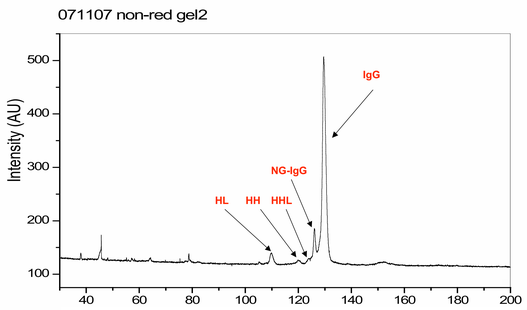


Figure 3. SDS-CGE separation of non-reduced IgG control standard with LINF detection.
IgG control standard was alkylated with iodoacetamide (IAM) and denatured at 70°C for 10 min. Separation was performed at 25°C in a 100cm, 50mm ID uncoated capillary, 85 cm to detection window. Injection was 10kV for 30 seconds. Separation voltage was 30 kV. LINF excitation was at 224nm and emission at 325nm.

Preparation of Dimer Containing IgG Test Sample

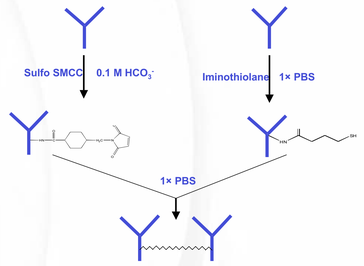


Figure 4. IgG cross-linking reaction using Beckman Coulter proprietary conjugation chemistry (F. Farooqui & MP Reddy, US Patent 6,942,972)
1) IgG was first activated with heterobifunctional cross-linker sulfo SMCC to attach a sulfhydryl-reactive maleimide group. 2 mg IgG 1 Kappa in 200 µl of bicarbonate buffer was activated with 200 µg of sulfo SMCC at room temperature for 1 hr. 2) Activate IgG with iminothiolane: 2 mg IgG 1 Kappa in 200 µl of 1 X PBS buffer was activated with 160 µg of iminothiolane at room temperature for 2 hr. 3) IgG cross-linking: Maleimaid-activated IgG was mixed with sulfhydryl-activated IgG and incubated at 4°C for 12 hr.

Separation of Dimer Containing IgG Test Sample Using SDS-CGE with LINF Detector

Baseline resolution of IgG monomer and dimer was achieved and stable baseline was obtained

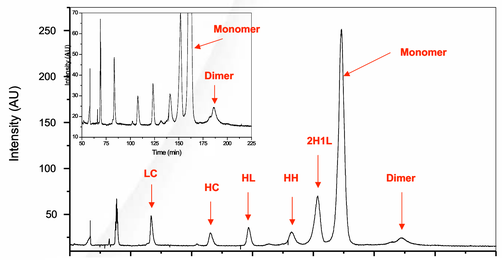


Figure 5. IgG dimer containing reaction mixture was alkylated with iodoacetamide (IAM) and denatured at 70°C for 10 min. Separation condition is the same as Figure 3.

Summary

- Improvement of sensitivity over UV detector was demonstrated using prototype LINF detector. The detection system* will be further optimized to maximum performance.
- The quantitative analysis results of LINF at 224 nm excitation appear consistent with those obtained using UV detection.
- A stable baseline was obtained in the high molecular weight region using the prototype LINF detector, enabling analysis of IgG aggregates.

* In development