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Boosting your sensitivity - Analysis of fluorescence-labeled proteins with semi-preparative FPLC and FLD

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SUMMARY

In many bioscience applications, fluorescence-labeled proteins are used to analyze cellular and biological functions. Here, fluorescein (FITC) and bovine serum albumin (BSA) were used as model molecules. We tested the integration of a fluorescence detector (FLD) into a semi-preparative FPLC system and evaluated the analysis of FITC labeled BSA in comparison with classical UV/VIS detection. The use of a fluorescence detector increases the sensitivity of the chromatographic analysis significantly.

INTRODUCTION

Fluorescence labeling of proteins is a versatile tool for a variety of research applications. By creating a labeled biomolecule this can be used for cell tracing, receptor labeling as well as in immunohistochemistry and cytochemistry. It helps analyzing biological structure, function and interactions of proteins and is used in many bioscience protocols. We used fluorescein (FITC) labeled bovine serum albumin (BSA) as a model protein. FITC is one of the most common fluorescent reagents for biological research because of its high absorptivity, excellent fluorescence quantum yield, and good water solubility. BSA is a protein derived from the blood serum of cows. It is often used for experiments due to its low cost, stability and broad availability. The protocol for labeling proteins involves a chromatographic purification step. Furthermore, fluorescence-labeled proteins can be as well used for chromatographic analysis as fluorescence detection responds selectively to compounds with a fluorophore and is more sensitive than UV/VIS detection. Here we integrated a fluorescence detector into a semi-preparative FPLC system and examined the analysis of a FITC labeled BSA comparing it to classical UV/VIS detection.

Additional Information

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RESULTS

A semi-preparative FPLC system was equipped with an additional fluorescence detector. The fluorescence detector has a biocompatible analytical flow cell which generates a back pressure due to its small inner diameter. First the back pressure of the system set up was analyzed. Water at room temperature generated a back pressure of around 1 bar for 1 mL/min and 2 bar at 2 mL/min. Increasing the flow rate will result in higher back pressure. Therefore we recommend to use flow rates below 2 mL/min if the fluorescence detector is used with pressure sensitive FPLC cartridges or columns. Special care should be taken by choosing the columns. The fluorescence detector can certainly be operated with pressure resistant columns. Additionally, the fluorescence detector should be used with a bypass valve in a semi-preparative system. By using this option, the fluorescence detector can be integrated into the flow in case of need **(Fig 1)**.

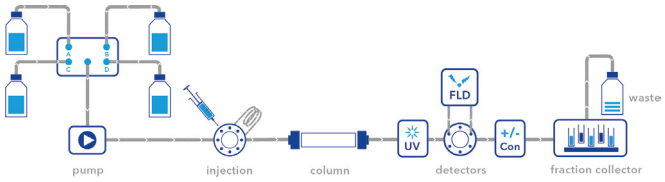


Fig. 1 Flow scheme of a typical semi-preparative FPLC system with fluorescence detector with bypass option.

FITC-BSA fluorescence signal was analyzed for 0.8 µg of sample 1 and 1.3 µg of sample 2 with the HIGH level mode **(Fig. 2)** The corresponding peak areas for the UV and fluorescence signal were determined, compared and the mean was calculated for the UV and fluorescence signal peak areas. Depending on the molar ratio of FITC and BSA the factors varied. The fluorescence signal peak area was between 24-fold and 29-fold higher than the peak area of the UV signal **(Tab. 1)**. Surprisingly higher functionalization shown by higher molar ratios F/P did not lead to higher factors comparing the fluorescence and UV 280 nm signal. An explanation for this might be quenching effects due to higher FITC functionalization. Since this phenomenon was not the scope of the work it was not further studied.

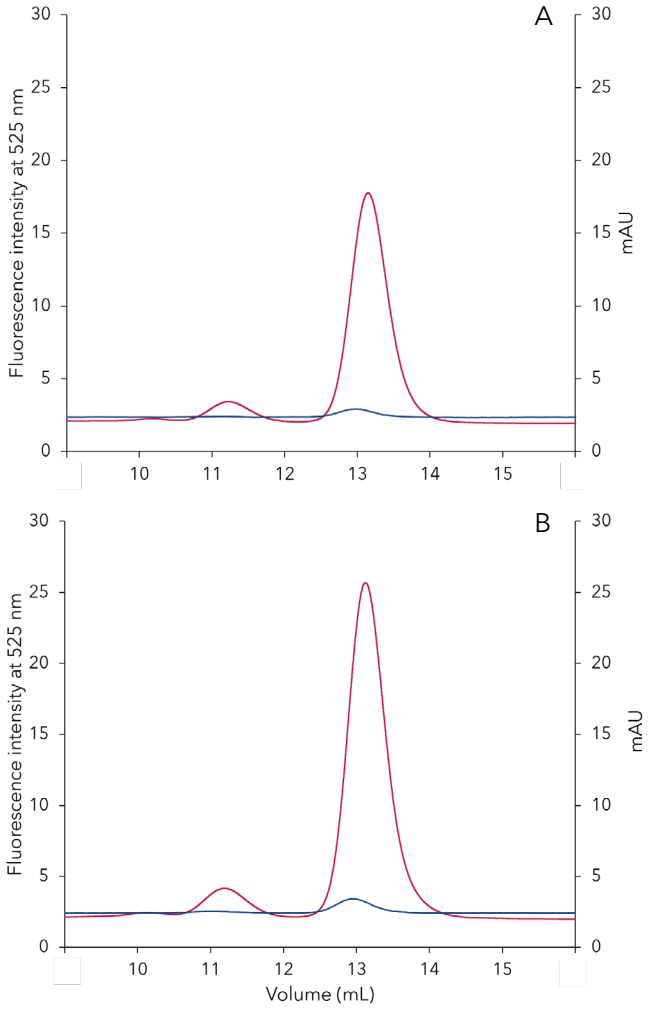


Fig. 2 Overlay of FITC-BSA peak measured with fluorescence (red) and UV 280 nm (blue). Sample 1 with 0.8 µg (A) and sample 2 with 1.3 µg (B).

Tab. 1 Factor of peak area increase comparing different FLD sensitivities

Sample	Concentration (µg)	FITC-BSA molar F/P	Mean peak area - FLD (HIGH level)	Mean peak area - UV 280 nm	Factor
1	0.8	1.13	505.6	21.1	24
2	1.3	0.3	349.6	12	29.1

SAMPLE PREPARATIONS

For the labeling of BSA with FITC, BSA was dissolved in 0.1 M Sodium carbonate pH 9 at a final concentration of 2 mg/mL. FITC was dissolved in DMSO at a concentration of 1 mg/mL. 1 mL of BSA solution was carefully mixed with 20 µL FITC solution. The labeling reaction took place unter two different conditions. The labeling was carried out at 4° C for 16 hours for sample 1 and at room temperature (RT) for 1.5 hours for sample 2. All solutions and samples were prepared fresh and the solutions containing FITC or BSA labeled with FITC were protected from light. After labeling BSA with FITC, the protein-dye solution was purified with a 5 mL Sepapure Desalting column (020X460SPZ) using PBS as buffer. A maximum of 1 mL sample was applied onto the column. The labeled protein was collected and the fluorescein/protein (F/P) molar ratio was determined using the following equation:

$$\text{Molar F/P} = \frac{MW_{BSA}}{MW_{FITC}} \times \frac{(A_{495} / E^{0.1\%\ FITC})}{[A_{280} - (0.35 \times A_{495})] / E^{0.1\%\ BSA}}$$

Molecular weight FITC	389
Molecular weight BSA	66,430
Absorption FITC $E^{0.1\%}$ *	195
Absorption BSA $E^{0.1\%}$ **	44,308.81
Correction factor FITC	0.35 x A_{495}

* Absorption at 490 nm at pH 13.

** Absorption at 280 nm of a protein at 1.0 mg/mL.

The absorbance of the conjugate sample was determined at 280 and 495 nm. For sample 1 a molar ratio of fluorophore per protein 1.13 and for sample 2 a molar ratio of 0.3 was calculated. As protein concentration determination by UV 280 nm is misleading, protein concentration was calculated from the used material. For the first labeling (sample 1) 8 mg BSA and for the second labeling (sample 2) 3 mg BSA were used. After purification a total volume of 9.6 mL for sample 1 and 3 mL for sample 2 was collected. From this a concentration reached 0.8 mg/mL for sample 1. 1.3 mg/mL were calculated for sample 2. For the first sample 0.8 µg and for sample 2 1.3 µg were injected. The FITC labeled BSA was analyzed with a KNAUER FPLC system including a fluorescence detector and a prepacked semi-preparative SEC column. Each run for each sensitivity level was measured minimum in duplicates.

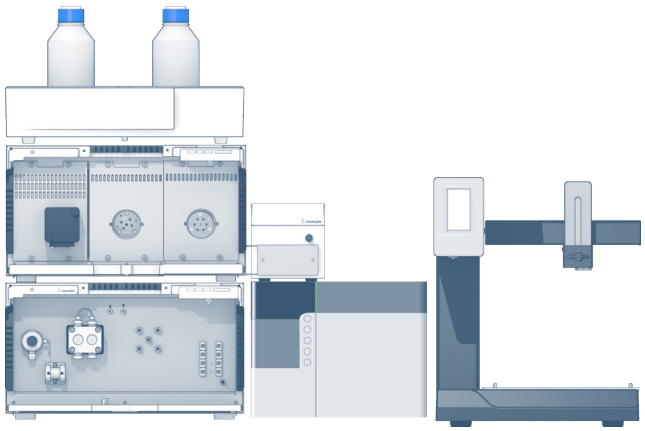
CONCLUSION

A semi-preparative FPLC system can easily be used with a fluorescence detector to analyze fluorescence-labeled proteins. The focus of the fluorescence detection is analytical. Special care should be taken by choosing the columns. An additional bypass valve should be integrated into the system to switch the fluorescence detector into the flow. The use of a fluorescence detector increased the sensitivity of the chromatographic measurement of fluorescence-labeled proteins in our study by factors between 24 to 29 fold. Thus, the use of a fluorescence detector increases the sensitivity of the chromatographic analysis significantly.

MATERIALS AND METHODS

Tab. 2 Method parameters

Buffer A	PBS (phosphate buffered saline, pH 7.4)
Gradient	isocratic
Flow rate	1.8 mL/min
Run temperature	RT
Injection volume	10 µL
Detection wavelength UV	280 nm
Excitation wavelength FLD	495 nm
Emission wavelength FLD	525 nm
Mode fluorescence detector	HIGH
System pressure	~ 42 bar
Run volume	min. 1 CV (24 mL)
Data rate	min. 2 Hz



Tab. 3 System configuration

Instrument	Description	Article No.
P6.1L	Metal-free, low pressure gradient FPLC pump with 10 mL ceramic pump head, degasser and 250 µL mixer	APH64EB
ASM 2.2L	**Left**: UVD2.1S variable single wavelength UV detector **Middle**: valve drive VU 4.1 **Right:** valve drive VU 4.1	AY00001
Flow Cell UV	Semi-preparative bio-compatible 3 mm UV Flow Cell, 1/16"	A4045
V4.1	Biocompatible multi-injection valve, 1/16"	AVN94CE
V4.1	Biocompatible two-position valve, 6 port	AVD24CE
CM2.1S	Conductivity monitor with flow cell for up to 100 mL/min flow rate	ADG30GD
RF20A	Fluorescence detector	A59200
Flow Cell FLD	Bioinert Flow Cell for fluorescence detector	A59212
Foxy R1	Fraction collector	A59100
Software	PurityChrom	A2650 A2652
Column	Prepacked SEC (10 x 300 mm) column for small-scale preparative purification, as well as for characterization and analysis of proteins with molecular weights (Mr) from 10,000 to 600,000, column volume (CV) 24 mL	