High sensitivity analysis of water-soluble, cyanine dye labeled proteins by high-performance liquid chromatography with fluorescence detection

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1. Introduction

In the post-genome sequencing era, protein analysis has been paid more and more attention. Up till now, various techniques have been employed, such as two-dimensional polyacrylamide gel electrophoresis (PAGE) [1], capillary electrophoresis (CE) [2,3] and high-performance liquid chromatography (HPLC) [4,5]. Unfortunately, the detection sensitivity of proteins still can hardly meet the requirements of real sample analysis. Most proteins with important biological function, such as the drug targets and biomarkers, are of extremely low concentration [6]. Therefore, the improvement on the detection sensitivity of proteins is an imperative task.

On-line sample concentration [7,8] has been regarded as a popular technique to decrease the LOD of analytes. Recently, such a technique was used for the on-chip pre-concentration of proteins, and about 600-fold improvement on the detection sensitivity was achieved [9,10]. Alternatively, fluorescence detection has been proved one of the most sensitive methods for protein analysis [11,12]. Although native fluorescence could be emitted by the aromatic amino acid residues, such as tryptophan, tyrosine and phenylalanine of some proteins [13], not all proteins could generate fluorescent signal. Therefore, the labeling of proteins with fluorescence reagents is indispensable to improve the detection sensitivity of proteins [14–16].

Up until now, various fluorescence reagents have been developed for protein derivatization. Fluorescein isothiocyanate (FITC) is one of the most commonly used reagents for protein labeling [17,18]. However, it is not only photo-bleached and pH sensitive, but also the required amount of proteins that could react with FITC is relatively large [19]. As a fluorogenic dye, 5-furoylquinoline-3-carboxaldehyde (FQ) was also used to derivatize native proteins, with LOD decreased to pM by CE with laser-induced fluorescence (LIF) detection [20]. However, cautions must be taken due to the necessity of KCN as the catalyst. In addition, as a large category of...
dyes, cyanines have been widely used in different fields [21], among which Cy2, Cy3, and Cy5 are usually used in two-dimensional fluorescence difference gel electrophoresis for comparison analysis [22,23]. However, the low water solubility of such dyes often results in protein precipitation during the derivatization procedures.

The active N-hydroxysuccinimide ester of 3H-Indolium,1-[(4-carboxyphenyl)methyl]-2-3-[1-[(4-carboxyphenyl)methyl]-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene]-1-propenyl]-3,3-dimethyl-5-sulfo-(9CI) (sb-cy3-NHS), is a water-soluble fluorescence reagent, first synthesized by our lab [24]. However, up till now, its application has not been reported. Here, to the best of our knowledge, for the first time it was used for the derivatization of proteins, which were further analyzed by HPLC with fluorescence detection. Through systematic optimization, the LOD of proteins could be decreased to nM level, much lower than those obtained by UV detection and fluorescence detection labeled by FITC. To demonstrate the suitability of sb-cy3-NHS, it was further applied for the derivatization of a protein mixture and egg white proteins, and proteins with low concentrations were successfully labeled, and detected with high sensitivity by HPLC.

2. Experimental

2.1. Materials and reagents

N-hydroxysuccinimide (NHS), trifluoroacetic acid and N,N′-dicyclohexylcarbodiimide (DCC) were purchased from Acros organics (Geel, Belgium). FITC, cytochrome c from horse heart, myoglobin from equine skeletal muscle, carbonic anhydrase from bovine erythrocytes, albumin human serum (HSA) and BSA were obtained from Sigma (St. Louis, MO). HPLC-grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). Microcon YM-3 with Ultracel YM-3 membrane was obtained from Millipore (Molsheim, France). All inorganic reagents were analytical-reagent grade, and others were of HPLC grade. A C8 column (5 μm, 300 Å, 4.6 mm × 250 mm) was ordered from Dalian Elite Analytical Instruments Co., Ltd. (Dalian, China). Fresh eggs were bought from a local supermarket. Water was purified by a Milli-Q system (Millipore, Molsheim, France).

2.2. Apparatus

Fluorescence spectra measurements were performed on a PTI-C-700 Felix and Time-Master system (PTI Technologies Inc., Oxnard, CA). HPLC experiments were performed on a system composed of an injection valve (Rheodyne, Cotati, CA), a four-line degasser, an intelligent pump equipped with quaternary gradient unit, an intelligent fluorescence detector (Jasco Co. Ltd., Tokyo, Japan) or a UV detector (Dalian Elite Analytical Instruments Co., Ltd., Dalian, China). The mobile phases were degassed by ultrasonication for 15 min before use. A paradigm GM4 μHPLC system (Michrom Bioreources Inc., Auburn, CA) with a homemade C18 column (300 μm i.d. × 50 mm), was coupled with an LCQDUO quadruple ion trap mass spectrometer (LCQ-IT MS, Thermo Fisher, San Jose, CA) for protein identification.

2.3. Microcolumn packing

The packing procedure of C18 microcolumn was as follows: in brief, C18 silica particle was firstly dispersed in bromoform to make slurry by ultrasonication for 5 min. Then the slurry was packed into a Peeksl tubing (300 μm i.d. × 50 mm) via homogenizer by an HPLC pump at about 6000 psi for 8 h. Finally, the column was conditioned by flushing with ACN for 2 h before use.

2.4. HPLC separation

HPLC separation of protein was carried out on a C8 column with binary mobile phase gradient. The column temperature was at 25℃. Eluent A was 100% water with 0.1% (v/v) TFA; eluent B was 95% (v/v) acetonitrile with 0.1% (v/v) TFA. The gradient for the separation of three-protein mixture was as follows: 0 min, 20% B; 30 min, 80% B; 40 min, 80% B; the flow rate was 1.0 mL min\(^{-1}\). The gradient for the separation of egg white proteins was as follows: 0 min, 25% B; 70 min: 60% B; 120 min: 80% B; the flow rate was constant at 0.25 mL min\(^{-1}\).

2.5. Synthesis of sb-cy3-NHS

The synthesis procedure of sb-cy3-NHS was referred to our previous report, as shown in Fig. 1a [24]. For sb-cy3, in brief, 1-(p-carboxybenzyl)-2,3,3-trimethylindolanium-5-sulfonate (718 mg, 2 mmol) was synthesized and refluxed after dissolution in pyridine (3 mL). Subsequently, triethyl orthoformate (0.9 mL, 6 mmol) was slowly added to the medium. The mixture was cooled and diluted with ether after heating for an additional 2.5 h. The resultant red precipitate was dissolved in methanol and reprecipitated with the addition of 2-propanol. Finally, the product
was filtered and dried under vacuum, and purified by RPLC with methanol–water as the mobile phase. The synthesized sb-cy3 (8 mg, 0.01 mmol) was then dissolved in dry DMF (2 mL). After the addition of N-hydroxysuccinimide (56 mg, 0.5 mmol) and N,N′-dicyclohexylcarbodiimide (21 mg, 0.1 mmol), the mixture was stirred at 25 °C for 2 days under a nitrogen atmosphere. After diluted with dry ethyl ether, the supernatant was decanted, washed repeatedly with dry ether, filtered under a nitrogen atmosphere and dried to powder. A nearly 100% yield of the active esters of sb-cy3, sb-cy3-NHS, was obtained.

2.6. Optimization on derivatization conditions

Various protein derivatization conditions were optimized with BSA as the model protein.

To study the effect of denaturant concentration on the derivatization, a 60 μL of 15 μM BSA (final concentration 5 μM) was mixed with 100 μL of guanidine hydrochloride solution with different concentrations. After each mixture was stirred at 50 °C for 30 min, 10 μL of 2 mM sb-cy3-NHS was added, and kept at 50 °C for derivatization. After 30 min, 5 μL of the derivatized protein solution was taken for further HPLC analysis. Unless otherwise stated, each sample was dissolved in 50 mM Na2CO3–NaHCO3 (pH 9.5).

Similarly, to optimize the effects of other experimental conditions, BSA was derivatized with sb-cy3-NHS respectively under the conditions of 0–2.5 h reaction time, 25–65 °C reaction temperature, pH 8.0–10.0 buffer, and 22–55 folds reagent excess in molar.

2.7. Protein derivatization

The derivatization of a three-protein mixture by sb-cy3-NHS was performed under the optimal conditions. A 60-μL solution of proteins, with the final concentration of 6.3, 1.1 and 40 μM for cytochrome c, BSA and myoglobin respectively, was mixed with 100 μL 4.5 M guanidine hydrochloride. After the mixture was stirred at 50 °C for 30 min, 10 μL of 4 mM sb-cy3-NHS was added, and then stirred at 50 °C for 30 min in dark. The sample was further diluted with 50 mM Na2CO3–NaHCO3 (pH 9.5) buffer by four folds, and then cooled to 25 °C. Subsequently, 10-μL aliquot of the derivatized protein mixture was injected, and further analyzed by HPLC. The derivatization procedure with FITC was carried out according to the reported optimal conditions [25], quite similar to that with sb-cy3-NHS except that 190 mM FITC was added, and the reaction was allowed to proceed at 25 °C for 2 h.

The egg white was carefully separated from the egg yolk by hand, and then transferred into a 50 mL Eppendorf tube at 4 °C. An aliquot of egg white was diluted with 50 mL PBS (pH 8.0), and the resultant suspension was ultrasonicated for 15 min at 4 °C. Then solid NaCl was added to the suspension to a final concentration of 1 M. After another 15 min ultrasonication at 4 °C, the sample was centrifuged at 12,000 rpm for 60 min to precipitate the insoluble proteins. The supernatant was collected and stocked as the soluble fraction at −20 °C. The protein concentration was determined by Bradford assay [26]. The derivatization procedures for egg white proteins respectively derivatized with sb-cy3-NHS and FITC were the same as those for the three-protein mixture.

2.8. Data analysis

Protein identification was performed using SEQUEST algorithm incorporated in BioWorks software and Swiss-Prot chicken protein database (Chicken). Peptides searched using fully tryptic cleavage constraints and up to two internal cleavages sites were allowed for tryptic digestion. The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. The SEQUEST results were filtered by Xcorr versus charge state. The peptides were considered as positive identification if Xcorr was higher than 1.9 for singly charged peptide, 2.2 for doubly charged peptide and 3.75 for triply charged peptide. ΔCn cutoff value was set to ≥0.1. Furthermore, the protein with two peptides identified was recognized as the true protein.

3. Results and discussion

The fluorescent reagent, sb-cy3-NHS, was designed with p-carboxybenzyl group on the nitrogen atoms for protein derivatization. As shown in Fig. 1b, the reagent could react with the amino groups of proteins (essentially side chains of lysine) to form covalent protein–dye complex [27]. Due to the presence of sulfonate groups and carboxyl groups, the reagent is water soluble. In addition, the p-carboxybenzyl group renders superior photo-stability to the conventional 5-carboxyalkyl-containing dye. Moreover, in comparison with isothiocyanate-containing dyes, e.g. FITC, the relatively high reaction activity of NHS-carboxyl group of sb-cy3-NHS permits proteins with low concentrations to be easily labeled under mild conditions.

3.1. Fluorescence spectrum of derivatized BSA

The fluorescence spectra of sb-cy3-NHS derivatized BSA was studied after diluted with H2O(CH3)2CN (50/50, v/v). From Fig. 2, it could be seen that the maximum excitation and emission wavelengths were respectively 554 and 572 nm. Within such a long-wavelength range, the interference of background fluorescence could be reduced, resulting in the high sensitive detection of biomolecules. Therefore, such two wavelengths were applied in the following experiments.

3.2. Effect of denaturant concentration

It is well known that the protein conformation has significant effects on derivatization. For native proteins, most amino groups are hidden in the interior. To ensure a complete reaction with the fluorescence reagents, it is necessary to denature proteins before labeling. Although various denaturants are available, such as DTT, urea and guanidine hydrochloride [28,29], by investigating the effect of denaturant on the protein derivatization with sb-cy3-NHS, it was found that guanidine hydrochloride was the most suitable one for protein derivatization using sb-cy3-NHS due to the minimum competition reaction with proteins. Therefore, systematic study on the effect of guanidine hydrochloride concentration on derivatization was studied. In our experiments, relative fluorescence response, defined as the ratio of each peak area to the largest
Fig. 3. Effect of denaturant concentration on derivatization.

one measured by HPLC in a normalized format, was used to evaluate the derivatization results. From Fig. 3, it could be seen that the relative fluorescence response was initially increased with the concentration of guanidine hydrochloride, and reached the maximum at 2.65 M, which might be caused by the complete exposure of reacting groups of proteins with sb-cy3-NHS. However, with the further increase of guanidine concentration, the response was gradually decreased, which meant that high salt might interfere with BSA derivatization by sb-cy3-NHS. Therefore, 2.65 M guanidine hydrochloride was selected to ensure high sensitive detection of proteins.

3.3. Effect of reaction parameters

The effect of reaction time on BSA derivatization was studied. From Fig. 4a, it could be seen that the relative fluorescence response of BSA increased sharply, and reached the maximum value within 0.5 h, which demonstrated that fast derivatization of proteins could be achieved with sb-cy3-NHS as the fluorescence reagent.

With 30 min derivatization time, the effect of reaction temperature was also studied. From Fig. 4b, it could be seen that the relative fluorescence response of BSA initially increased with temperature, and reached the maximum at 50°C, which meant that elevated temperature might be helpful to accelerate the reaction. However, with further increase of temperature, the relative fluorescence response decreased. Through studying the thermal stability of sb-cy3-NHS, it was found that the stability of such a dye began to decrease when over 50°C. Therefore, the decreased fluorescence response of derivatized BSA was mainly generated by the instability of fluorescence reagent. Therefore, 50°C was selected as the optimal reaction temperature.

Fig. 4. Effect of reaction parameters on derivatization: (a) reaction time, (b) reaction temperature, (c) pH, and (d) reagent molar excess.
eluent A, 100% water with 0.1% TFA; eluent B, 95% acetonitrile with 0.1% TFA; gradient conditions: 0–30 min: 20–80% B; flow rate, 1.0 mL min⁻¹; the column temperature, 25 °C.

The pH value of reaction buffer affects protein derivatization as well. From Fig. 4c it could be seen that the maximum fluorescence intensity was obtained at pH 9.5. The deprotonation of primary amino groups at this pH might facilitate the interaction with sb-cy3-NHS. Consequently, pH 9.5 was selected as the optimal pH value of the derivatization buffer.

In addition, the molar ratio of fluorescence reagent to protein is another important parameter that affects the reaction speed. From Fig. 4d, it could be seen that when the ratio reached 39, the highest relative fluorescence response was obtained. However, with further increased molar ratio, due to fluorescence quenching, the signal intensity began to decrease. Accordingly, 39 times excess in molar ratio was chosen in our following experiments.

3.4. Reproducibility of derivatization

Under the above-mentioned optimal conditions, 2.65 M guanidine hydrochloride for protein denaturing, 39 times excess of fluorescent reagent (mole ratio) and reaction at 50 °C for 30 min, the derivatization reproducibility of BSA with sb-cy3-NHS was studied by HPLC with fluorescence detection. In five consecutive derivatization runs, the RSDs of the retention time and the peak area were respectively 0.35 and 1.06%, and those for 6 consecutive days were 0.87 and 1.38%. All these data indicated the good reproducibility of protein derivatized by sb-cy3-NHS.

Furthermore, no noticeable decrease of fluorescence signal was observed when the derivatized BSA was analyzed by HPLC after stored in the reaction buffer under 4 °C for 1 month, which further demonstrated the high stability of protein derivatized by sb-cy3-NHS.

3.5. Depletion of excess fluorescence reagent for HPLC analysis

To achieve complete derivatization, 39 times excess of sb-cy3-NHS was added, which might seriously interfere with the separation and detection of proteins by HPLC. Fig. 5a shows the analysis of excess fluorescence reagent blank by HPLC with fluorescence detection. Besides the peak of sb-cy3-NHS, other peaks might be the hydrolysis products. Therefore, it is necessary to deplete the residual fluorescence reagent.

Up till now, some off-line techniques have been developed, including gel filtration [30], affinity [31], dialysis [32] and extraction [33], which not only are labor-intensive and time-consuming, but also might cause sample loss. In our experiments, owe to the good hydrophilicity of sb-cy3-NHS and it’s hydrolyzing products, a facile on-column removal technique was developed by flushing with the mobile phase composed of 20% ACN (v/v). As shown in Fig. 5b, the peaks of excess fluorescence reagent were almost disappeared. Since mobile phase with high ACN concentration is necessary to elute proteins, the depletion of excess sb-cy3-NHS is beneficial for protein detection.

3.6. Derivatization of standard proteins

Because of the relatively high absorptivity and excellent fluorescence quantum yield [34], FITC is one of the most popular fluorescent labeling reagents, and widely used for the covalent labeling of proteins. In our experiment, five proteins (cytochrome c, myoglobin, carbonic anhydrase, HSA, and BSA), with molecular mass ranging from 12.4 to 69.2 kDa, were derivatized respectively by FITC and sb-cy3-NHS under individually optimized conditions. The LODs were measured with UV and fluorescence detection. It should be noted that the LODs with fluorescence detection refer to the minimum amount of proteins that could be derivatized with a detection signal-to-noise ratio of 3 [20].

From Table 1, it could be seen that the LODs of five standard proteins by fluorescence detection labeled by FITC were all at nM level, similar to UV detection, which was also proven by the previous report [11,35]. The main reason was that the derivatization of primary amines by FITC was slow and inefficient. However, for sb-cy3-NHS derivatization, the LODs of proteins were down to nM level. Especially for BSA, it reached 12.8 nM, nearly 100-fold lower than that with UV and FITC labeled fluorescence detection.

![Fluorescence reagent](image)

**Fig. 5.** Chromatograms of excess sb-cy3-NHS before (a) and after (b) on-column depletion. Chromatographic conditions: column: 250 mm × 4.6 mm, C8 5 μm, 300 Å; eluent A, 100% water with 0.1% TFA; eluent B, 95% acetonitrile with 0.1% TFA; gradient conditions: 0–30 min: 25–60% B; 70–120 min: 60–80% B; flow rate: 0.25 mL min⁻¹; the column temperature, 25 °C.

![Chromatograms](image)

**Fig. 6.** Separation of a three-protein mixture by fluorescence (labeled by sb-cy3-NHS (a) and FITC (b)) and UV (c) detection. Chromatographic conditions: column: 250 mm × 4.6 mm, C8 5 μm, 300 Å; eluent A, 100% water with 0.1% TFA; eluent B, 95% acetonitrile with 0.1% TFA; gradient conditions: 0–30 min: 20–80% B; 30–40 min: 80–80% B; flow rate, 1.0 mL min⁻¹; the column temperature, 25 °C. Peaks: 1, cytochrome c (1.6 μM); 2, BSA (0.28 μM); 3, myoglobin (10 μM).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight (kDa)</th>
<th>UV (μM)</th>
<th>Fluorescence detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>12.4</td>
<td>2.8</td>
<td>285</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>17.8</td>
<td>2.0</td>
<td>99.0</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>31</td>
<td>1.1</td>
<td>28.5</td>
</tr>
<tr>
<td>HSA</td>
<td>66.5</td>
<td>0.5</td>
<td>26.5</td>
</tr>
<tr>
<td>BSA</td>
<td>69.2</td>
<td>1.0</td>
<td>12.8</td>
</tr>
</tbody>
</table>
results indicated that the detection sensitivity of proteins derivatized with sb-cy3-NHS could be obviously improved, displaying greater superiority in the labeling of low concentration proteins.

3.7. Derivatization of protein mixture

A three-protein mixture, including cytochrome c, BSA and myoglobin, was derivatized with sb-cy3-NHS and commercial FITC, respectively, and then analyzed by HPLC after diluted to certain concentrations. All proteins labeled by sb-cy3-NHS were detected (Fig. 6a) with higher sensitivity than those derivatized by FITC (Fig. 6b). The low protein concentration and thus less derivatization efficacy might be the main cause for the low responses when derivatized by FITC. In addition, it was noteworthy that due to the high hydrophobicity of FITC, the on-column depletion technique was invalidated to remove the excess FITC. Therefore, it was removed by filter membranes (MW cutoff 3000 Da). After the off-line filter removal of 12 h, the leftovers were still existed, observed as two peaks. Furthermore, the high hydrophobicity of FITC increased the hydrophobicity of labeled protein, resulting in the lagged retention time of myoglobin (Fig. 6b). For UV detection, the same protein mixture was analyzed by HPLC, and only the high concentration myoglobin was detected (Fig. 6c). These results further demonstrate that sb-cy3-NHS is of great advantages for the derivatization of proteins with low concentration.

To evaluate the efficiency of sb-cy3-NHS to label real samples, egg white proteins were labeled. As shown in Fig. 7a, under the optimum conditions, the fluorescence intensity of 1.75 μg extracted proteins derivatized by sb-cy3-NHS was greatly enhanced compared to those labeled by FITC (Fig. 7b) and direct detection by UV (Fig. 7c). Two representative fractions in Fig. 7a were collected with a large volume injection (ca. 40-fold), which were further digested by trypsin and analyzed by μRPLC-ESI-MS/MS. Two proteins, 0807280A ovomucoid (MW = 20,198.4) and A Chain A, binding of N-acetylglucosamine to chicken (MW = 14,314.1), were identified from peak 1, as shown in Fig. 7a1, and another two proteins, A Chain A, diferric chicken serum transferrin at 2.8 (MW = 75,828.6) and B Chain B, loop-inserted structure of P1-P1’ cleaved (MW = 42,725.7), were identified from peak 2, as shown in Fig. 7a2. All these results further suggested that the high sensitivity detection of proteins could be easily achieved after sb-cy3-NHS derivatization and detection by HPLC with a fluorescence detector, especially for those with low concentration.

4. Conclusion

In this work, sb-cy3-NHS was used for protein derivatization. Not only the long excitation and emission wavelengths could effectively reduce the background interference of biological samples analysis, but also the high hydrophilicity of such a dye enabled the facile
depletion of excess reagents by RPLC. In addition, the good stability of such a dye ensured the good reproducibility of sample derivatization. Furthermore, proteins with concentration at the level of nM could be efficiently labeled by sb-cy3-NHS. All these results demonstrated that sb-cy3-NHS was a good derivatization reagent to achieve high sensitive detection for low concentration proteins.

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References

[25] The derivatization procedure with FITC used in this study was a standard one that based on the product information of Molecular Probes (Invitrogen, Carosbad, CA), Website: http://www.probes.invitrogen.com/media/pis/mp06434.pdf.