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## Dansyl-labeled heterobifunctional crosslinker with NHS-ester and protected sulfhydryl groups

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### Abstract

Introduction of a fluorescence chromophore into bifunctional crosslinkers is designed to assign two different functions. Normal crosslinker properties together with a fluorescence signal for quantification. As an example of this type of fluorescent-labeled crosslinker, dansyl-labeled heterobifunctional crosslinker with an NHS ester and sulfhydryl group was synthesized. The application of the synthesized crosslinker was demonstrated by conjugation of bovine serum albumin (BSA) to the surface of maleimide-containing liposome.

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### Introduction

Modification of proteins, peptides, and other biopolymers with ligands or reporter molecules is an important tool in targeting drug delivery, immunology, histochemistry, and cell biology [1-6]. Chemical crosslinking of two bioactive components can be most easily achieved with bifunctional, especially, heterobifunctional compounds [7-8]. The bifunctional reagents used for such coupling reactions are, therefore, identified as crosslinkers, and are commercially available.

Since most bioactive elements such as proteins have multifunctional groups that can be used for either modification or conjugation, preservation of the optimal biological and chemical characteristics of the elements in the conjugate and the final product is often controlled by selecting the optimal ligand density range, or degree of modification. Consequently, the accurate determination of conjugated components is critical for the crosslinker applications. Because of the lack of convenient and suitable alternatives, currently employed methods to determine the amount of ligand coupled in the conjugate offer only qualitative results in most cases. They are more tedious and often affected by many factors (such higher background, no absorption peak and no suitable functional group).

In order to use crosslinkers to conjugate two bioactive elements and also easily to quantify the accurate degree of modification or conjugation, we designed a series of fluorescent-labeled crosslinkers. In the first report here, a dansyl-labeled amino acid (dansyl-lysine), was reacted with N-succinimidyl S-acetylthioacetate (SATA) and then with NHS to produce a new fluorescent-labeled heterobifunctional crosslinker with a carboxylic NHS ester at one end and a protected sulfhydryl at the other end (Fig. 1). By introducing a fluorescent-moiety to crosslinkers, one can preserve the normal crosslinker's properties and also be able to quantify the degree of modification and conjugation. In addition, a fluorophore introduced



1ml methanol, to this solution 200 ul of triethylamine was added to make the solution basic. 100 mg (0.433 mmol) of SATA was dissolved in 1 ml of chloroform. The SATA solution was slowly added into dansyl-L-lysine solution at room temperature, and the mixture was stirred for one hour. The reaction was monitored by TLC (CHCl<sub>3</sub>/MeOH/AcOH, 85:15:0.2), R<sub>f</sub> values are dansyllysine (0.024), SATA (0.81), dansyl-ATA-lysine (0.29). The Dansyl-ATA-lysine was produced after aqueous work up to be a yellow powder (75%Yield). The Dansyl-ATA-lysine (85mg) and NHS (29mg) were dissolved in CH<sub>2</sub>Cl<sub>2</sub>, and DCC (46mg) then slowly added. The reaction mixture was stirred at room temperature overnight. The precipitated DCU was filtered and the filtrate was diluted with 50 mL of chloroform, washed with water and dried over anhydrous sodium sulfate. Removal of the solvent from the dried extracts followed by recrystallization of the residue from ethanol yielded 61.2mg (60% yield) of the Dansyl-ATA-lysine-NHS.

**Modification of BSA Using Dansyl-ATA-lysine-NHS.** BSA protein (5 mg) was dissolved in 1mL of 0.1 M NaHCO<sub>3</sub> at pH 8.3. To this solution Dansyl-ATA-lysine -NHS (0.5 mg in 200 m l DMF was added, and the mixture was stirred at room temperature for 1 h. The modified BSA was purified through a 1.0 x 10 cm Sepharose CL-6B column equilibrated with HBS buffer at pH 7.5. The protein concentration was determined by the micro BCA assay. The fluorescent-crosslinker covalently linked to BSA was measured from the absorbance at 343 nm ( dansyl moiety). The degree of modification was calculated to be 3.4 crosslinkers per mol of BSA. The modified BSA was dried in a lyophilizer and stored at -20°C.

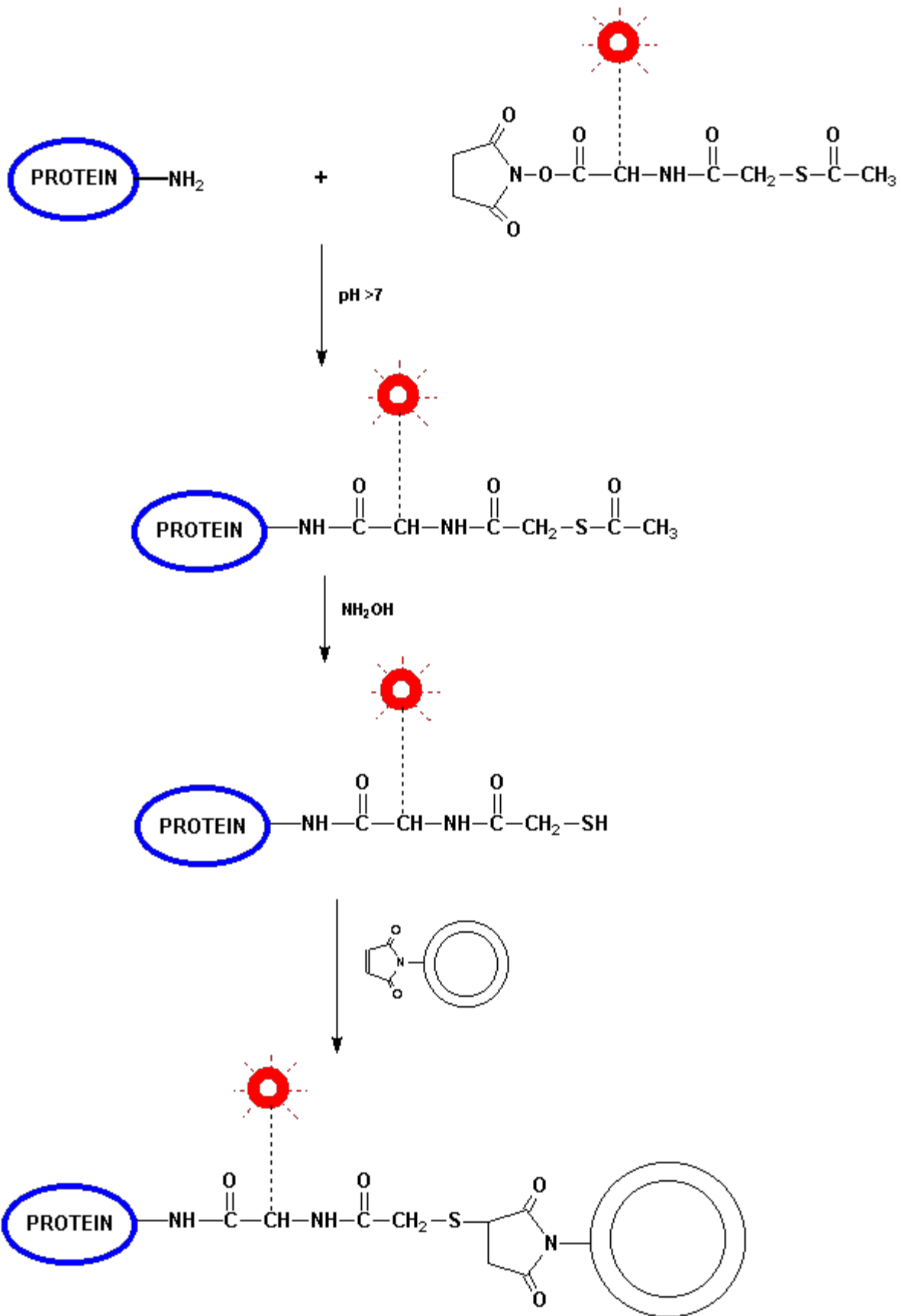
**Conjugation of Modified BSA to MPB-liposomes.** Liposomes [EPC/CHOL/MPB-DSPE, 53:45:2] or [EPC/CHOL, 55:45] were prepared as described previously by Hope et al. [9] The extruded MPB-LUVs were used for the conjugation reaction, and the MPB-free-LUVs were used as a control. Modified BSA (1mg) was hydrolyzed in 0.25 ml of 0.1M NaOH for 1 h at room temperature to produce actived BSA-Dansyl-SH. The hydrolysis reaction was stopped by passing the mixture down a Bio-gel B10 column with HBS buffer and the modified BSA concentration was determined from the absorbance at 343 nm. The purified BSA-Dansyl-SH was incubated with MPB-LUVs or MPB-free-LUVs (control) at a 1:5 ratio (protein to LUV) at room temperature overnight. The coupling mixture was separated by a Sepharose CL-6B column equilibrated with HBS buffer.

**Protein Assays.** BSA was quantified using the micro BCA protein assay kit from Pierce. A set of BSA standards containing 2-20 m g of protein and samples were diluted with 5% Triton X-100 and sufficient distilled water to produce a total volume of 1.0 mL. 1.0 mL of working reagent was then added. The mixed solutions were vortexed and incubated at 60°C for 1 h. The protein concentrations of the samples were calculated by relating to the standards from the absorbance at 562nm.

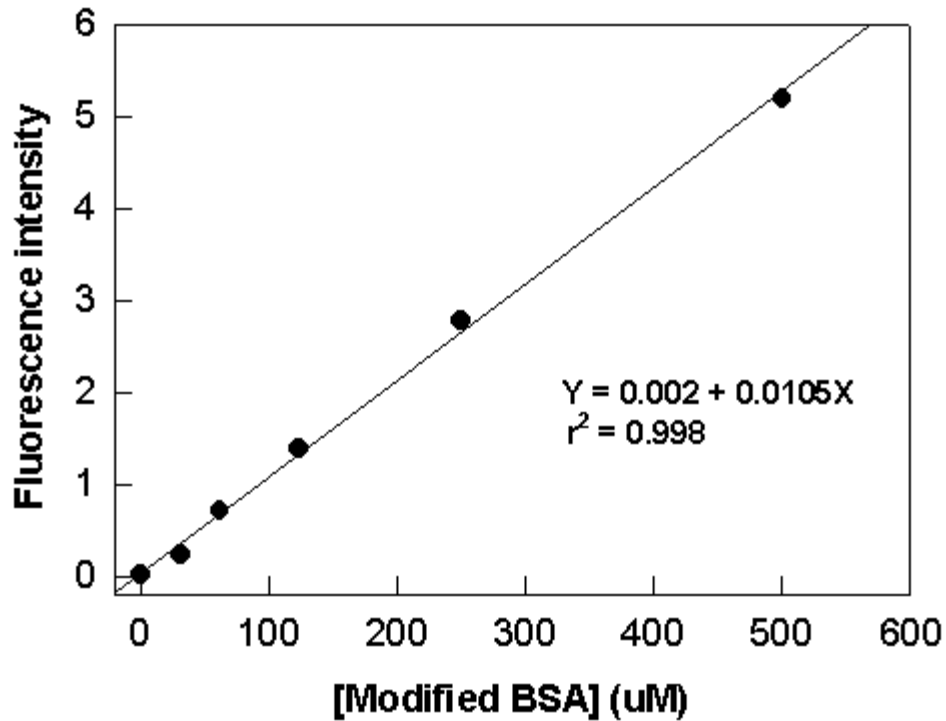
## Results and Discussion

The synthetic approach for the new fluorescence heterobifunctional dansyl-labeled-crosslinkers is outlined in Fig. 1. Reaction of Dansyl-lysine with SATA gave in high yield Dansyl-ATA-Lysine. The final product, succinimidyl ester, was then obtained via a DCC mediated esterification with NHS.

The Dansyl-labeled heterobifunctional crosslinker with NHS ester and sulfhydryl groups retained the crosslinker property of SATA. As an example shown in Fig. 2, this crosslinker allows us to covalently conjugating BSA protein to the surface of MPB-liposomes. The crosslinker is reacted first with protein amino groups, and then hydrolytically cleaved to generate thiol moieties for covalently coupling protein with MPB-liposomes. The fluorophore in modified protein is stable and produces a quantitative fluorescence. Therefore, the concentration of the modified protein in solution (with or without coupling) can be quantified by comparison with the appropriate standard curve.(Fig. 3).



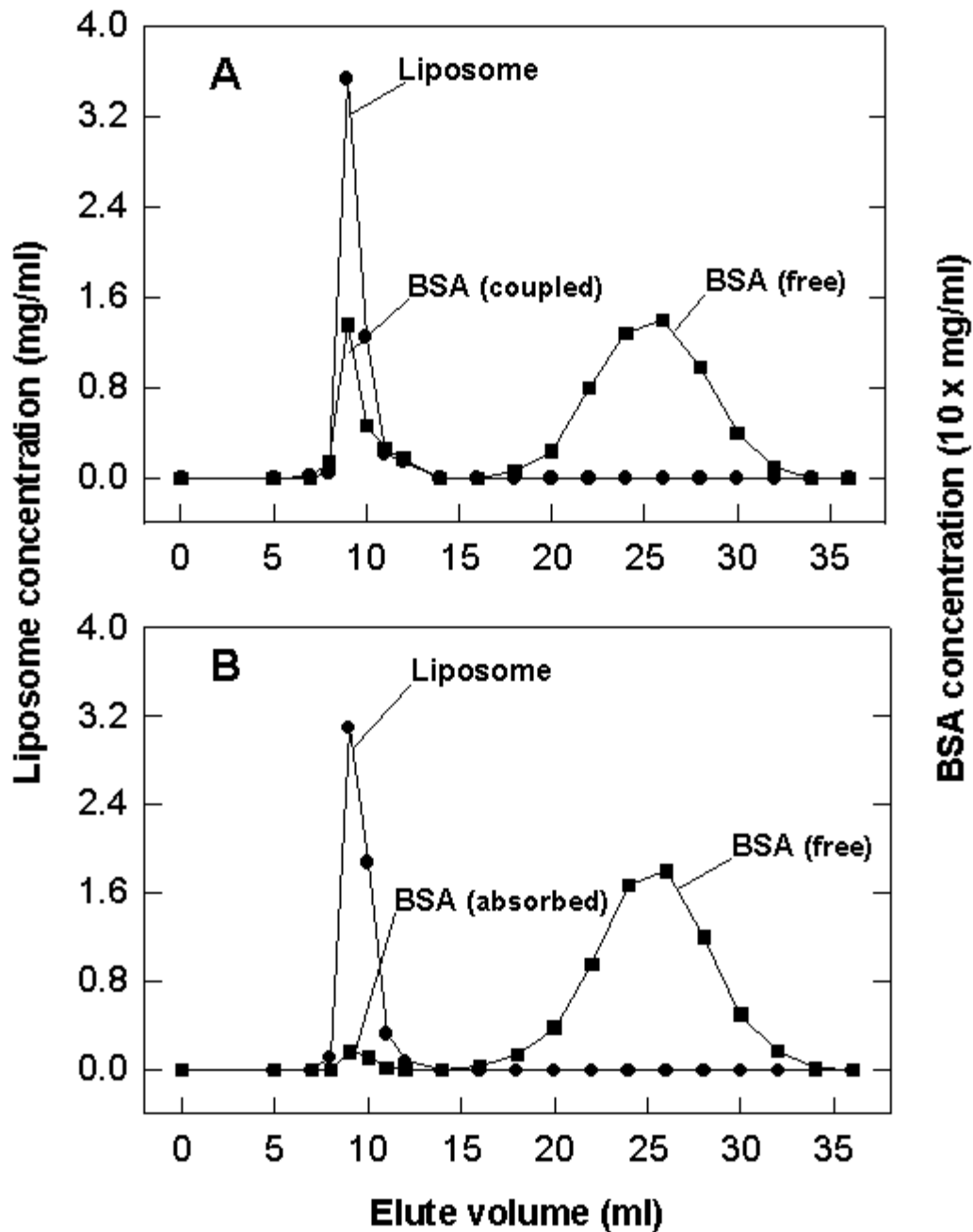
**Fig. 2.** An example of application of the fluorescent-labeled crosslinker for conjugation of protein onto MPB-liposome.



**Fig. 3.** Linear regression analysis of standard curve for the dansyl-ATA-lysine modified BSA.

Fig. 4 shows the elution profiles of the protein-coupled LUVs and the protein-absorbed LUVs on a Sepharose CL 6B column. It is clear that the fluorescence detected in the liposome fraction is due to covalent conjugation since a control experiment (MPB-free-liposome) in the same reaction condition only gave very low amount fluorescence intensity. The similar results of protein on the liposomal surface determined by dansyl fluorescence and by the micro BCA protein assay further confirms the reliability of protein quantification by fluorescence measurement (Table 1).

In summary, a new heterobifunctional crosslinker has been synthesized and its usefulness for bioconjugation has been demonstrated by BSA protein modification and conjugation of the modified BSA to MPB-liposomes.



**Fig. 4.** Elution profiles obtained following coupling of modified BSA to the surface of MPB-LUVs. Liposome were monitored using a radiolabelled cholesteryl hexadecyl ether [ $^3\text{H}$ ]CHE, and BSA protein was determined by the dansyl fluorescence intensity at 515nm, bandpass 8nm (excitation at 353, bandpass 2nm). (A). coupling reaction with MPB-liposome; (B). control reaction with MPB-free-liposome.

**Table 1. Comparison of BCA and Fluorescence Assays for Quantification of Protein conjugated onto MPB-liposomes<sup>a</sup>.**

Entry	Liposome	Degree of conjugation (m g protein/mg lipid)	
		BCA assay	Fluorescence assay
1	MPB-LUVs	37	36.7
2	LUVs	2	6

a. The coupling reaction was carried out at protein to LUV ratio of 1:5 in HBS buffer overnight.

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