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Notes & Tips

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ABSTRACT

This study revealed a major interference from sulfo-*N*-hydroxysuccinimide (sulfo-NHS) in the bicinchoninic acid (BCA) protein assay. Sulfo-NHS, a common reagent used in bioconjugation and analytical biochemistry, exhibited absorbance signals and absorbance peaks at 562 nm, comparable to bovine serum albumin (BSA). However, the combined absorbance of sulfo-NHS and BSA was not strictly additive. The sulfo-NHS interference was suggested to be caused by the reduction of Cu^{2+} in the BCA Kit's reagent B (4% cupric sulfate) in a manner similar to that of the protein.

The bicinchoninic acid (BCA)[1] protein assay [1] is widely used for determination of protein concentrations ranging from 25 to 2000 μg/mL. As a water-soluble sodium salt, BCA exhibits high sensitivity and specificity for Cu^{1+}. Similar to the Biuret reaction, the presence of protein in an alkaline medium reduces Cu^{2+} to Cu^{1+}. Consequently, a purple-colored reaction product is formed by the chelation of two molecules of BCA with each Cu^{1+}, which exhibits a strong absorbance at 562 nm. The total protein concentration is reflected by the sample color change from green to purple in proportion to a given protein concentration. The color formation caused by the reaction of protein with BCA is dependent on its number of peptide bonds, its macromolecular structure, and the availability of four amino acids (cysteine, cystine, tryptophan, and tyrosine) in the protein [2]. The amino acid sequence, p*I*, and the presence of certain side chains or prosthetic groups can also affect the color formation significantly. The extent of the color formation is determined by more than the sum of individual color-forming functional groups [2].

The Thermo Scientific BCA protein assay kit's instruction (Cat. No. 23227) indicates several potential interfering substances such as ascorbic acid, catecholamine, creatinine, cysteine, EGTA, impure glycerol, hydrogen peroxide, hydrazides, iron, lipids, melibiose, phenol red, impure sucrose, tryptophan, tyrosine, and uric acid. Phospholipids [3], glucose [4], mercaptoethanol [4], and other substances such as acetamidophenol, 3,4-dihydroxyphenylalanine, dithiothreitol, glutathione, and penicillamine [2] also interfere with the BCA assay. There is also a temperature-dependent reaction of peptide bonds with Cu^{2+} that can increase the concentration of Cu^{1+}. There are numerous reports where proteins have been quantified by the BCA protein assay in the presence of sulfo-NHS [5–13]. However, the interference of sulfo-NHS in the BCA assay has not been reported.

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Thermo Scientific) and sulfo-NHS (Thermo Scientific)-based heterobifunctional crosslinking of proteins is one of the most widely used procedures for biomolecular conjugation [14–16] and biochip-based assay development [17–19]. Our results in this study show the interference of sulfo-NHS with the BCA protein assay, which can lead to inaccurate protein estimation. To our knowledge, this is the first report on the interference caused by sulfo-NHS in the BCA assay.

Identical varying concentrations (25–2000 μg/mL) of sulfo-NHS, EDC, and EDC–sulfo-NHS were employed in the BCA protein assay in the absence of protein. The absorbance at 562 nm, the characteristic wavelength corresponding to the absorption peak of the purple-colored complex, was then compared with that of the same concentration of BSA. Fig. 1A shows a very pronounced interference caused by sulfo-NHS in the BCA protein assay, whether it was employed alone or with EDC. In contrast, EDC alone did not show interference with the BCA protein assay. Note also that the absorbances obtained at 562 nm were very similar for sulfo-NHS, EDC–sulfo-NHS, and BSA. The BCA assay was also performed with

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[1] *Abbreviations used:* BCA, bicinchoninic acid; BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; sulfo-NHS, sulfo-*N*-hydroxy succinimide.

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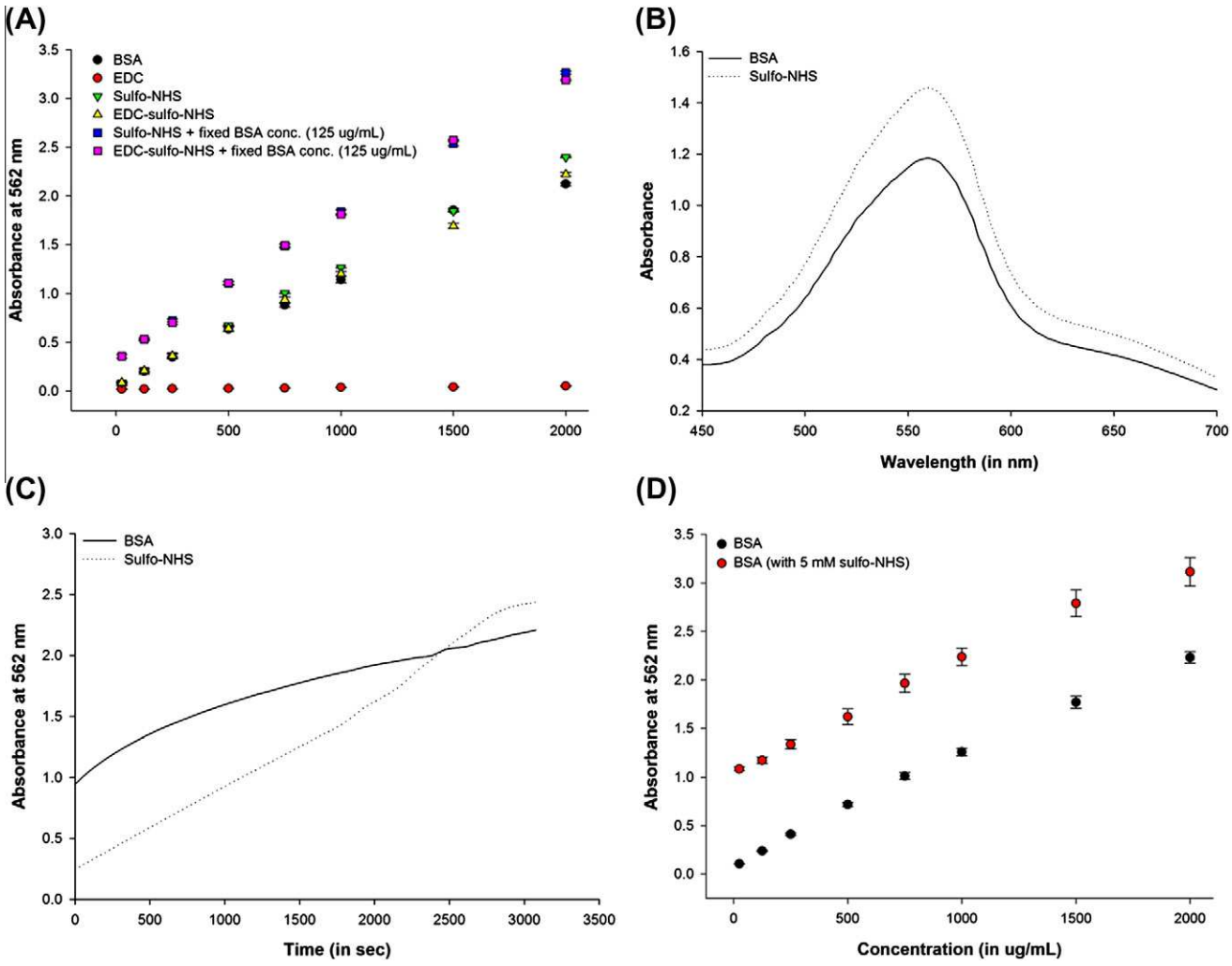

Fig.1. (A) BCA protein assay curves with varying concentrations (25–2000 μg/mL) of BSA, EDC, sulfo-NHS, EDC-sulfo-NHS, and sulfo-NHS/EDC–sulfo-NHS mixed with a fixed BSA concentration of 125 μg/mL. (B) Absorbance scan from 450 to 700 nm of the purple-colored complexes produced after BCA protein assays with BSA/sulfo-NHS (1 mg/mL). (C) Time-response measurements at 562 nm of the BCA protein assay solutions with BSA/sulfo-NHS (1 mg/mL). (D) The BCA standard curve for BSA in the presence and absence of 5 mM sulfo-NHS.

identical varying concentrations of sulfo-NHS and EDC–sulfo-NHS but each concentration mixed with a fixed BSA concentration (125 μg/mL). The combined absorbance of sulfo-NHS/EDC–sulfo-NHS (varying concentrations) and BSA (125 μg/mL) at 562 nm was not additive and was greater than the sum of the absorbances for the various concentrations (25–2000 μg/mL) of sulfo-NHS/EDC–sulfo-NHS and the fixed BSA concentration.

Sulfo-NHS and BSA displayed the same absorption maximum of 562 nm (Fig. 1B) as identified from the absorbance scan from 450 to 700 nm obtained with respective purple-colored complexes in the BCA protein assay after 30 min of incubation at 37 °C, suggesting the formation of the same chromophore. The time-response kinetic measurement (Fig. 1C) was then performed with the BCA protein assay solution immediately after the addition of BCA reagent to understand the behavior of the colorimetric reaction with BSA and sulfo-NHS. The ambient temperature of about 27 °C inside the microtiter plate reader (Tecan, Infinite M200 Pro) was used for these experiments, different from the actual BCA assay measurements done at 37 °C. The initial absorbance value and the initial reaction rate of BSA with BCA were higher but showed a gradual decrease with time. This can be explained by the fact that the initial reaction occurs from the interaction of copper and BCA with cysteine, tryptophan, and tyrosine residues of BSA and subsequently, the peptide bond is also responsible for color development. On the other hand, the reaction rate for sulfo-NHS showed consistent single-phase kinetics.

A study was conducted for the interference of EDC with the Lowry method of protein determination [20], where EDC and BSA showed the same absorbance peak. The interference was attributed to the reduction of the Folin–Ciocalteu reagent by EDC. However, there was no interference from EDC with the BCA assay in our present study. The interference of phospholipids with the BCA assay was reported to produce a similar absorbance peak as BSA but the combined absorbance of protein and phospholipid was not strictly additive [3]. Similarly, glucose, mercaptoethanol, and dithiothreitol also produced the same absorbance response as BSA and an identical absorbance peak at 562 nm [4].

In the present study, the interference from sulfo-NHS can be effectively managed by plotting a BCA standard curve for BSA in the presence and absence of 5 mM sulfo-NHS (Fig. 1D). The mean average difference in the absorbance values of BSA in the presence and absence of sulfo-NHS, which is actually the signal contributed due to the presence of sulfo-NHS in BSA solution, is calculated. Subsequently, the true absorbance of an unknown protein sample can then be determined fairly well by subtracting out the contribution of absorbance derived from the sulfo-NHS.

The Thermo Scientific BCA kit's instructions suggest that changing the BCA working reagent A:B ratio of 50:1 to 50:2 or 50:3 may

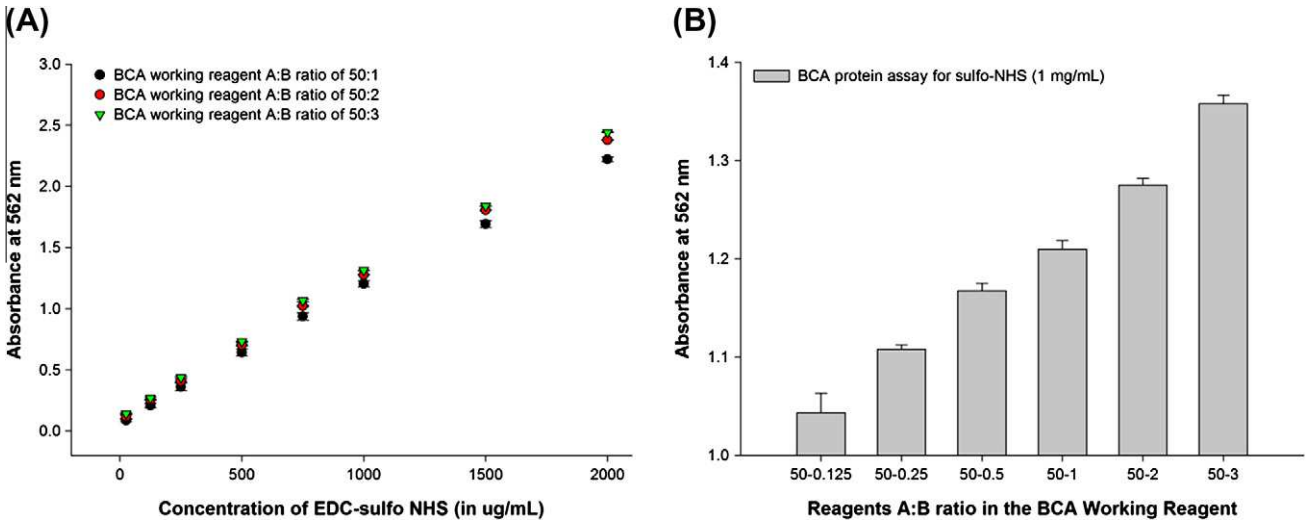

Fig.2. (A) The BCA protein assay with varying sulfo-NHS concentrations (25–2000 μg/mL) using BCA working reagent (WR) A:B ratio of 50:1 (normal procedure) to 50:2 or 50:3. (B) The BCA protein assay for sulfo-NHS (1 mg/mL) with increased concentrations of Cu^{2+} in the BCA working reagent, i.e., increasing the concentration of reagent B and keeping the reagent A concentration fixed.

eliminate interferences by copper-chelating agents owing to increasing copper in the BCA working reagent. However, there was a slight increase in the absorbance values from varied concentrations of the sulfo-NHS in the BCA assay (Fig. 2A) when the Cu^{2+} concentration (BCA Kit's reagent B, i.e., 4% cupric sulfate) was increased, thereby implying that sulfo-NHS itself was not a copper-chelating agent and its interference was likely due to the reduction of Cu^{2+} in a similar fashion as that caused by the protein. Therefore, the BCA protein assay was also performed with a fixed and sufficient sulfo-NHS concentration (1 mg/mL) in the presence of increasing concentrations of Cu^{2+} in the BCA working reagent, i.e., increasing the concentration of reagent B but keeping the concentration of reagent A fixed. The colorimetric absorbance values (Fig. 2B) were directly proportional to the Cu^{2+} concentration in the BCA working reagent. These results suggest that sulfo-NHS interferes with the BCA protein assay by reducing Cu^{2+} to Cu^{1+}, which then reacts with BCA to form a BCA–Cu^{1+} complex and hence, the increased absorbance values.

In summary, sulfo-NHS has remarkable interference with the BCA protein assay, which is suggested to be caused by the reduction of Cu^{2+} to Cu^{1+} and resulting in producing an absorbance peak at 562 nm in the BCA working reagent. However, the combined absorbance of sulfo-NHS and BSA was not strictly additive. Our present findings would be of greater utility to the researchers, relying on the BCA protein assay for the estimation of protein concentration, as they can prevent the potential errors resulting from the presence of sulfo-NHS along with the protein in their specific bioanalytical applications.

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