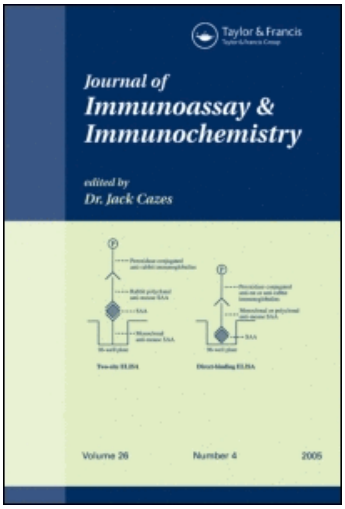

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Yannis Dotsikas[a]; Yannis L. Loukas[a]
[a] Department of Pharmaceutical Chemistry, School of Pharmacy, University of Athens, Panepistimioupoli Zografou, Athens, Greece

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Application of Avidin-Biotin Technology for the Characterization of a Model Hapten-Protein Conjugate

Yannis Dotsikas and Yannis L. Loukas
Department of Pharmaceutical Chemistry, School of Pharmacy, University of Athens, Panepistimioupoli Zografou, Athens, Greece

Abstract: A simple method was developed for the rapid characterization of the covalent binding of haptens to proteins such as enzymes, bovine serum albumin (BSA), and other carrier-proteins and antibodies. In the present study, a commercially available fentanyl-BSA conjugate was characterized by a 4′-hydroxyazobenzene-2-carboxylic acid (HABA) dye assay that followed a biotinylation reaction. This protocol allowed the indirect observation of the average hapten number per BSA molecule. Such measurement is useful for optimizing reaction conditions to yield a more precisely defined product for immunological applications. The obtained result was within the limits suggested by the manufacturer of the conjugate.

Keywords: Hapten-BSA conjugate, Electrospray mass spectrometry, HABA, Biotin

INTRODUCTION

The chemical derivatization of proteins to form bioconjugates is an extremely important process in the field of immunology. The conjugation of haptens and protein-carriers with the use of various cross-linking reagents results in the formation of specifically derivatized macromolecules. For example, immunoconjugates comprised of haptens linked to carrier proteins are useful for eliciting the production of specific antibodies for use in immunoassays, and hapten-modified enzymes form the basis of many types of enzyme-immunoassays. Furthermore, the same process is followed in order to bind a label on a protein (e.g., carboxyfluorescein and antibody).

Address correspondence to Yannis L. Loukas, Department of Pharmaceutical Chemistry, School of Pharmacy, University of Athens, Panepistimioupoli Zografou, GR-157 71, Athens, Greece. E-mail: loukas@pharm.uoa.gr

The reactive groups in proteins that are commonly targeted for cross-linking include: primary amines in lysine residues and terminal amino groups, carboxylate groups in aspartic and glutamic acid residues and terminal carboxylic acids, and sulfhydryl group in cysteine side-chains. Various methods are available for coupling ligands to functional groups on proteins.[1,2] The preferred residue for attachment is lysine,[3] since modification of other functional groups frequently leads to protein inactivation.[4] The available fentanyl-BSA conjugate was synthesized according to previously described protocols[5–7] that result in conjugation through the primary amino groups of the protein.

A number of analytical methods have been described that allow the determination of an average hapten number. Direct UV absorbance measurements have been used when the ε_{max} of the introduced chromophore is distinct from that of the protein.[8] UV difference spectroscopy provided an alternative method when there was spectral overlap.[9] When the derivatizing agent is doped with a radiolabeled analog, the extent of modification can be followed directly by measuring the associated radioactivity.[10] Assay for the unreacted amino groups on the protein either indirectly, by trinitrobenzenesulfonic acid (TNBS) titration,[11] or directly, by amino acid analysis,[12] is an additional method of bioconjugate characterization.

Another means of bioconjugate characterization relies on the measurement of the molecular weight of the modified protein. Until recently, only low-resolution methods such as SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)[13] or isoelectric focusing[14] were available. Lately, mass spectrometry techniques like ESI, Fast Atom Bombardment (FAB) and Matrix-Assisted Laser Desorption Ionization (MALDI) have emerged as powerful tools for directly observing the masses of modified proteins.[15,16] ESI-MS is a soft ionization technique which typically gives only the parent ion with little or no fragmentation under normal conditions.[17] Unlike MALDI-MS, samples are introduced as solutions in a combination of water and organic solvents, which is particularly convenient for the analysis of proteins,[18] DNA/RNA[19] and other macromolecules.

In the present study, a commercially available fentanyl-BSA conjugate was used and the average number of haptens covalently bound through the primary amines was estimated by applying a HABA dye assay, that followed a biotinylation reaction. The latter was performed after all free primary amines of the conjugate reacted with a biotinylation reagent solution to give biotinylated derivatives. The degree of biotinylation was compared with a fully biotinylated non-derivatized BSA and led to an indirect estimation of the average number of fentanyl molecules per BSA molecule.

EXPERIMENTAL

Materials

Fentanyl-BSA conjugate (M.W. ~74,000) was obtained from Biostride Inc. (USA) and, according to the manufacturer, 16–22 molecules of fentanyl

were bound onto each BSA molecule through primary amines (lysine residues and terminal amino groups). Sulfo-NHS-LC-Biotin, a water-soluble biotinylation reagent, from Molecular Probes (Eugene, OR). BSA (RIA and ELISA grade) was brought from Calbiochem (Germany). HABA, avidin, biotin, and all other reagents and solvents were from Sigma (St. Louis, MO). All aqueous solutions and buffers were prepared using water, deionised and doubly distilled with a Millipore Milli-Q Plus System (Resistivity >18 MΩ cm).

Instruments

HABA dye assay and other UV/Vis measurements were carried out by using a Perkin-Elmer Lamda 7 UV/Vis spectrophotometer at room temperature.

The LC/MS spectra of protein solutions were measured with an AQA LC/MS system (Finnigan, Thermoquest, France) equipped with an electrospray ionization (ESI) source and a quadrupole mass analyzer. BSA and fentanyl-BSA conjugate solutions (0.1 mM) in water/methanol/acetic acid (48/48/4) were transferred from AQA reservoir to ESI probe via a fused silica capillary by pressurizing the reservoir. A pressure of approximately 6 psi was needed to produce the desired flow rate of 10 μL/min. The probe heater temperature was set at 130–140°C and probe and cone voltage were held at 3500 and 40 V, respectively.

Biotinylation Reaction

Biotin, a 244 Dalton vitamin, can be associated with many proteins without altering the biological activity of the protein. The glycoprotein chicken egg white avidin and its non-glycosylated homologue, streptavidin, which is produced by the soil bacterium *Streptomyces avidinii*, have become important functional proteins, owing to their extensive use in affinity-based separations and diagnostic assays, as well as for a variety of other applications that have collectively become known as avidin-biotin technology.[20] The avidin-biotin interaction is the strongest known noncovalent biological recognition (approximate $K_a = 10^{15}\ M^{-1}$) between protein and ligand.[21]

Sulfo-NHS-LC-Biotin, a water-soluble biotinylation reagent, was used in specific molar ratios to biotinylate BSA and fentanyl-BSA conjugate for 2 hours. The reaction of primary amines with the biotinylation reagent (*N*-hydroxysulfosuccinimide ester) is best at neutral pH values and above. In the present study, a phosphate buffer pH = 7.2 was used. Under these conditions, the amine reacts with the biotinylation reagent by nucleophilic attack, as indicated in Fig. 1.

The excess biotinylation reagent was removed by gel filtration on a 1 × 13 cm Sephadex G-25 column preequilibrated with phosphate buffer saline, pH = 7.4 (PBS). The biotinylated fentanyl-BSA conjugates were eluted first and these fractions were pooled after their absorbance was measured at 280 nm.

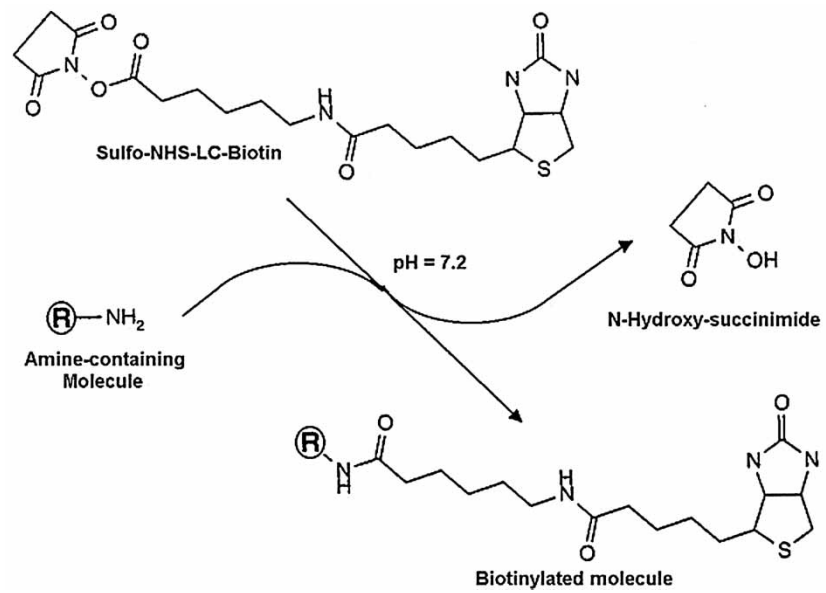

Figure 1. Biotinylation reaction of a protein molecule with the use of the biotinylation reagent Sulfo-NHS-LC-Biotin via nucleophilic attack.

Determination of the Level of Biotinylation

The method is based on the finding that the dye HABA, when bound to avidin, yields a characteristic absorption band at 500 nm. In the absence of biotin, the dye is capable of forming noncovalent complexes with avidin at its biotin-binding sites. The addition of biotin to this complex results in displacement of HABA from the binding site, since the affinity constant of the biotin-avidin interaction is much greater than the one for avidin-HABA (approximate $K_a = 6 \times 10^6\ M^{-1}$). As HABA is displaced, the absorbance of the complex decreases proportionally.

Thus, the amount of biotin present in the solution can be determined by plotting the avidin-HABA absorbance at 500 nm versus the absorbance modulation with increasing concentrations of the added biotin (Fig. 2). Since a biotinylated molecule is able to interact with avidin at its biotin-binding sites just as strongly as biotin in solution, the degree of biotinylation may be determined using the HABA method as well. In the present study, the HABA method was applied according to a previously described protocol.[22]

In brief, the standard curve indicated in Fig. 2 was constructed by measuring the absorbance at 500 nm of a 3 mL avidin solution (0.5 mg/mL) plus 75 μL of a HABA dye solution (10 mM) in PBS, pH = 7.4. Next, specific aliquots of the biotin solution (0.5 mM) were added, repetitively, to the avidin-HABA solution and mixed well after each addition, followed by

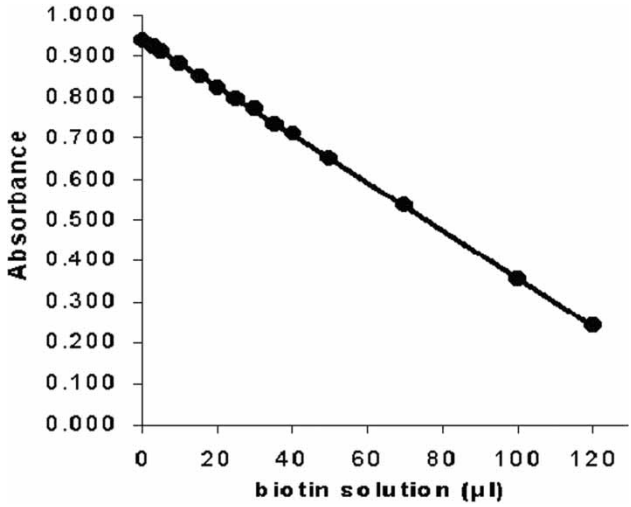

Figure 2. HABA dye plot showing the decrease in absorbance at 500 nm of an avidin-HABA solution in the presence of increasing amounts of biotin solution. The linear equation that describes this plot is the following: Absorbance $= -0.0058$ (μL biotin solution) $+ 0.9414$ (R $= 0.9999$).

the measurement of the resultant absorbance. The absorbance readings were plotted against the amount of biotin added, enabling one to determine the amount of biotin present in the protein sample, after adding a small amount of the biotinylated protein in a novel avidin-HABA solution.

RESULTS AND DISCUSSION

ESI Spectra of BSA

The general utility of ESI-MS for characterizing proteins is well known. The spectrum of BSA (Fig. 3) provides important information with regard to the protein. The multiply charged ions observed in the positive ion spectrum are produced primarily as a result of proton attachment to "available" basic sites in the protein molecule. The availability of ionizable basic sites is determined by the conformation of the protein under the conditions of study. In general, a protein in a tightly folded conformation will have fewer basic sites available for protonation compared to the same protein in an unfolded conformation.[23] In conclusion, the gas-phase ions observed in the electrospray mass spectrum reflect the charge states of the protein in solution, providing data for the accessible amines that participate in a chemical reaction, e.g., with the biotinylation reagent.

As shown in Fig. 3, a broad range of charge states is observed in the spectrum of BSA, yielding a substantial redundancy of data for the

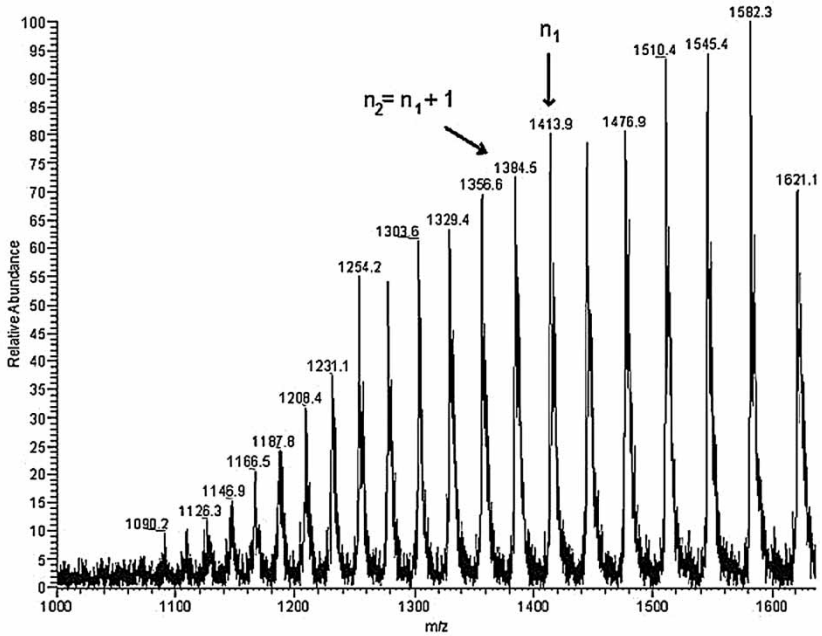

Figure 3. ESI mass spectrum of BSA in MeOH/H_2O/CH_3COOH 48/48/4.

determination of the molecular weight and the maximum charge state. If a positive ion series is assumed to represent different protonation states, then the mass/charge ratios X_1 and X_2, of adjacent members of the ion series are given by

$$X_1 = (M + nH)/n \tag{1}$$

and

$$X_2 = [M + (n + 1)H]/(n + 1) \tag{2}$$

where M is the molecular mass, n the charge state, and H the proton mass. Solving these equations gives

$$n = (X_2 - 1)/(X_1 - X_2) \tag{3}$$

and

$$M = nX_1 - n \tag{4}$$

allowing the estimation of n ($n = 47$) and M ($M = 66{,}406$). That means that m/z value X_1 indicates a charge state of $+47$. The maximum charged state is indicated by the m/z value of 1090.2 and it can be estimated by transforming the previous equations, giving a charge state of $+61$.

Previous researchers[11] have found that the total number of basic amino acids (lysine, histidine and arginine) per molecule of BSA is 100. These basic groups, plus the terminal amino group, are responsible for the characteristic peaks observed in the BSA spectrum. The number of the available lysine groups for conjugation with haptens is smaller than 61 and was calculated with the HABA dye assay.

Calculation of the Average Number of Fentanyl Molecules per BSA

The average degree of conjugation will be calculated by using the HABA dye assay that was previously described. Firstly, a molar ratio 1:61 of BSA to biotinylation reagent was used in a biotinylation reaction of the unconjugated BSA. The ratio 1:61 was used since 61 amino groups were recorded by ESI-MS. By applying the linear equation indicated in Fig. 2, 32 biotin groups were calculated to be incorporated per molecule of BSA. This number shows the lysine groups plus the terminal amino groups that can react with the biotinylation reagent. Considering that fentanyl has a quite similar M.W. with biotin and it was covalently bound into the same reacting groups of the protein, the average fentanyl groups per BSA molecule in the present sample can be indirectly determined by coupling the non-conjugated primary amines with biotin groups which can be easily measured.

A 1:32 molar ratio of fentanyl-BSA conjugate (0.5 mg) to biotinylation reagent was used according to the biotinylation protocol which was previously described. The application of the linear equation of Fig. 2 gives a number of 12 biotin groups (n_{biot}) per BSA molecule, indicating that, in average, the number of haptens (n_{hap}) can be calculated using the following equation:

$$n_{hap} = n_L - n_{biot}, \quad (\text{where } n_L = 32 \text{ for BSA}) \tag{5}$$

which gives a number of 20 fentanyl molecules incorporated into each protein molecule. This value was within the limits set by the manufacturer of the product (16–22 haptens per BSA), enhancing the reliability of the combination of ESI-MS with a HABA dye assay in characterizing such complicated protein molecules. The biotinylated fentanyl-BSA conjugate described in this study has been used as a label in the development of very sensitive fentanyl enzyme immunoassays.[24,25]

Equation (5) can be applied to characterize other hapten-BSA derivatives in which the hapten has reacted with the same protein reactive groups and has a size that is similar to biotin; otherwise, the method is not suitable. Furthermore, this method is directly applicable, not only for BSA, but for other carrier proteins too, if the maximum lysine groups in the protein surface is well known. If not, the procedure previously described can provide a rapid estimation of n_L.

CONCLUSIONS

In summary of this study, ESI-MS and HABA dye assay appear to be a very reliable combination for characterizing BSA bioconjugates. ESI-MS was able to detect the molecular weight and the maximum charged state of the unconjugated BSA, which indicates the available amine groups of the protein. Furthermore, ESI-MS provided information for the appropriate molar ratio of protein to biotinylation reagent for the biotinylation reaction, while an HABA dye assay allowed an accurate estimation of the average hapten number per BSA. The hapten density of the conjugate is often cited as an important property. In light of ever increasing quality and regulatory demands, the characterization of bioconjugates has become critically important; the present study provides a simple, rapid and reliable combination of methods for derivatives in which the hapten has been attached through the lysine residue.

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