

# Determination of heroin hapten densities in protein conjugates: comparison of MALDI-TOF MS, TNBS and indirect Ellman assay

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### Novel Aspect

Comparison of MALDI-TOF high molecular weight measurement accuracy in predicting drug conjugates densities to two established colorimetric techniques: trinitrobenzenesulfonic acid (TNBS) and Indirect Ellman assay

### Introduction

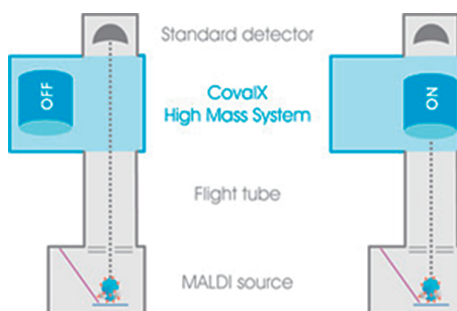
One strategy for treating drug addiction is the development of vaccines directed against drugs of abuse. Vaccines induce antibodies which bind to the drug in the blood, preventing it from crossing the blood-brain barrier, thereby, blocking the euphoria and addictive effects. Drugs of abuse are small molecules which do not induce antibodies. To induce antibodies, a surrogate drug (MorHapten) is attached to a carrier protein. Evaluation of

the number of haptens attached to the carrier is a vaccine characterization requirement. Evaluation was done using indirect methods, trinitrobenzenesulfonic acid (TNBS) to measure amines, and indirect (modified) Ellman assay to measure linkers, and a direct method, High Mass (HM) MALDI TOF MS. The goal of this study is to compare indirect methods to MALDI-TOF MS.

### Methods

BSA was treated with NHS-(PEG)<sub>2</sub>-maleimide linker at varying molar ratios. Following 2 hour incubation, excess linker was removed by gel filtration. Aliquots of BSA-maleimide were mixed with excess MESNA for 5 minutes. Ellman's reagent (160 uL, 10 mM) was added and the absorbance was monitored at 412 nm. The remainder of BSA-maleimide was treated with 100-fold excess of hapten. After 2 hours, excess hapten was removed by dialysis. The BSA and BSA-hapten were treated with TNBS. The solution incubated (37°C, 2 hours) and monitored at 420 nm. Desalted BSA and BSA-hapten (0.5uL) were

mixed with 0.5 ul of sinapinic acid. A MALDI capable of ultra high mass analysis (MegaTOF, Shimadzu) was used to monitor these haptens up to the megadalton level. The AXIMNA MegaTOF features the CovalX detector, which offers increased sensitivity when measuring masses over 100 KDa. Its design incorporates an Ion-Conversion Dynode (ICD) coupled to a secondary electron multiplier. This design allows the generation of secondary ions from colliding high molecular weight primary ion, which are reaccelerated into a secondary electron multiplier.



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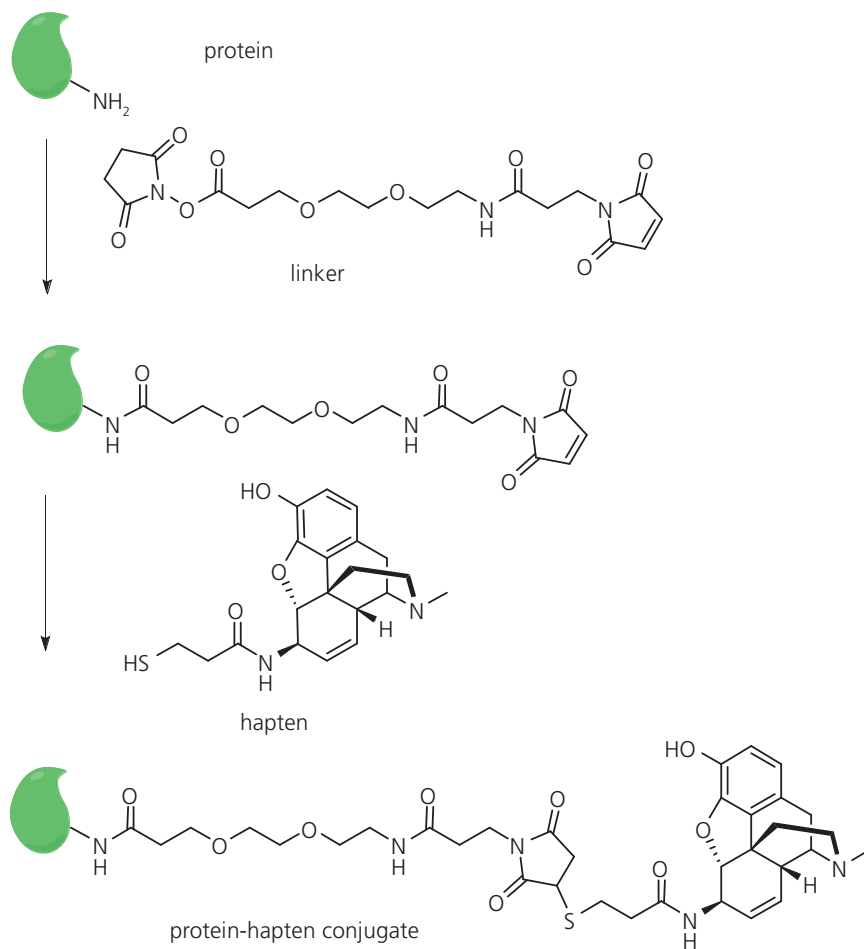


Figure 1. Summary of the coupling procedure. Free primary amines from the immunogen (BSA) are coupled to a linker (NHS-(PEG)<sub>2</sub>-maleimide). The protein linker complex is then reacted with a compound very similar in structure to the drug of abuse of interest. In this study, a synthetic molecule called hapten (MorHap) was used. It has a very similar structure to heroin.

## Results

The conjugation of heroin hapten to BSA was optimized by monitoring the number of attached haptens at various linker to BSA protein ratios (5, 10, 25, 50, 100, 200 and 400).

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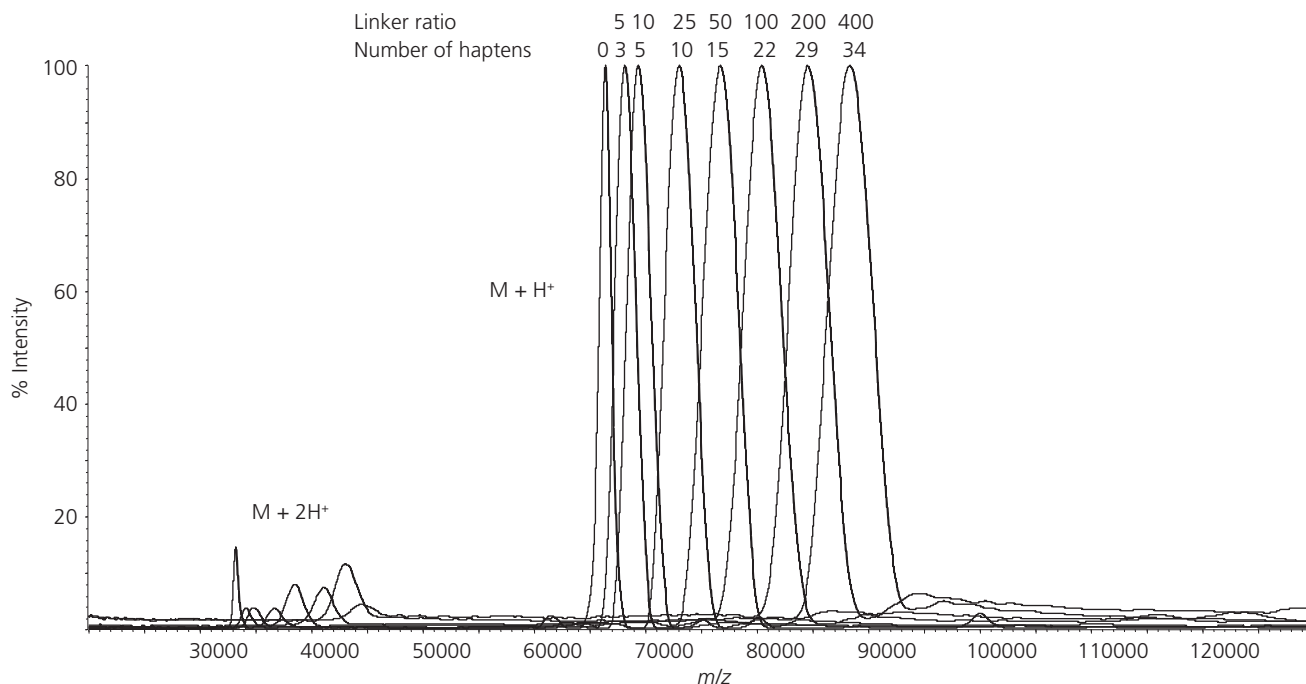


Figure 2. Overlay of MALDI-TOF MS spectra. Eight spectra are displayed representing the mass measurement of the BSA-MorHap complex at increasing linker to BSA ratios. The first peak represents the mass measurement of the starting material. The next peaks resulted from mass measurement of the BSA-MorHap complex at linker to BSA ratios of 5, 10, 25, 50, 100, 200 and 400. The excess of MorHap was a 100 fold.

Table 1. Table summarizing Morhap equivalence. Each mass spectrum was acquired three times. MorHap equivalence was deduced for each run.

Linker to BSA ratio	MorHap equivalence			Average number of Morhap attached (standard deviation)
	Run 1	Run 2	Run 3	
5	2.6	3	2.9	2.8 (0.2)
10	4.6	4.4	4.8	4.6 (0.2)
25	9.8	9.7	9.4	9.6 (0.2)
50	15.2	14.3	14.9	14.8 (0.5)
100	22.1	22	22.5	22.2 (0.3)
200	27.5	28.3	28.9	28.2 (0.1)
400	33.4	35	35.1	34.5 (0.9)

$$\text{MorHap Eq} = \frac{\text{Mass}_{\text{BSA-MorHap}} - \text{Mass}_{\text{BSA}}}{\text{Mass}_{\text{MorHap-linker}}}$$

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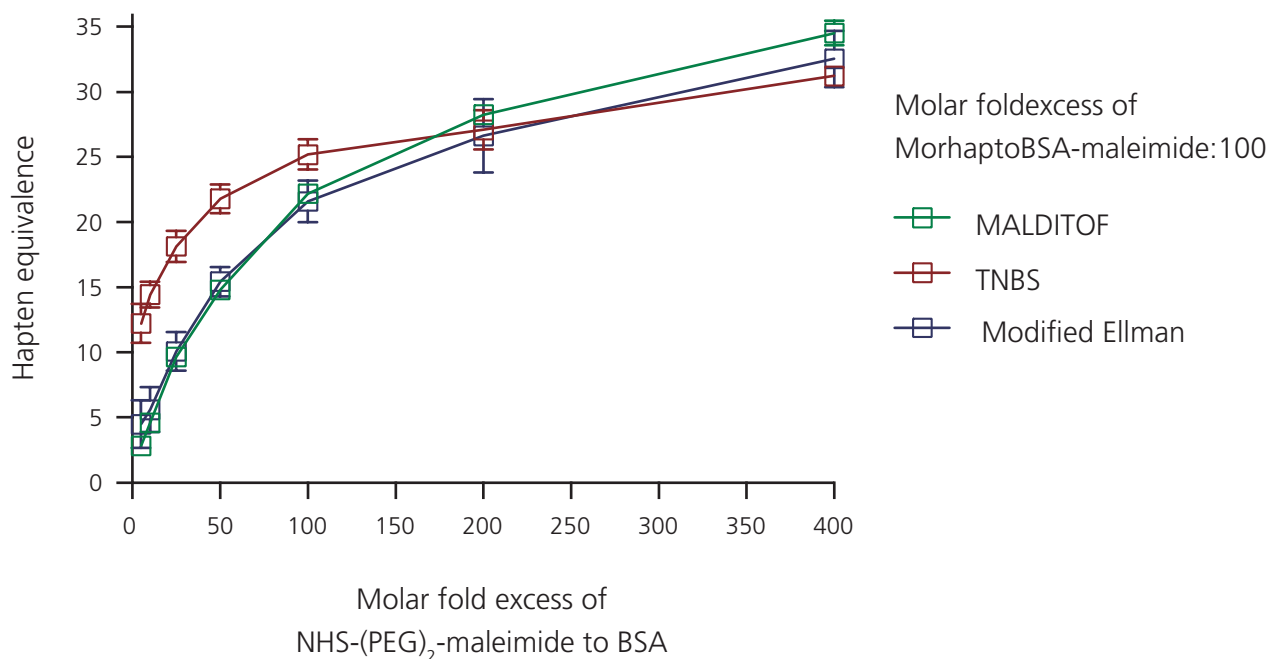


Figure 3. Graphic plots comparing MALDI-TOF, TNBS and Modified Ellman methods for a molar excess of MorHap to BSA-linker 100. The curves generated for TNBS and MALDI were practically overlapping. Modified Ellman, however, tend to overestimate the hapten density at lower molecular weight. Similar results were obtained with molar excess of MorHap to BSA-maleimide of 25 and 400.

Table 2. Expected average experimental time for drug-linker density determination.

Expected experimental time		
HM-MALDI-TOF	TNBS	Modified Ellman
15 mins	180 mins	60 mins

## Discussion

Determination of the number of MorHap drug-linker covalent attachment to BSA was performed using three different techniques. Plots showed similar trends for various MorHap excesses. The maximum number of attached MorHap are in accordance with the number of surface Lysine available (30 to 35). The attachment efficiency increases exponentially when increasing molar excess of NHS-(PEG)<sub>2</sub>-Maleimide Linker. The attachment slows down when reaching higher molar excess probably

due to steric hindrance and the lack of available sites. Modified Ellman and MALDI-TOF estimations strongly agree with each other. However, TNBS seems to over-estimate the number of attached MorHap at lower excess of NHS-PEG. This may be due to conformational changes induced in the protein and/or a masking effect of the non-modified lysines by the neighboring haptens obstructing the reaction with TNBS, which resulted in the overestimation of haptens.

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### Conclusion

This is the first report comparing three different methods for evaluation of drug-linker covalent attachment to a protein host. Two of the three techniques strongly agree with their estimation: modified Ellman and MALDI-TOF MS.

TNBS showed similar numbers at higher molar excess of linker. Due to the ease of use and speed of acquisition and sensitivity, this study makes a strong case for MALDI-TOF as a tool of choice for protein-drug conjugates analysis.