Bioconjugate Chemistry

Communication

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Simple Radiometric Method for Accurately Quantitating Epitope Densities of Hapten–Protein Conjugates with Sulfhydryl Linkages

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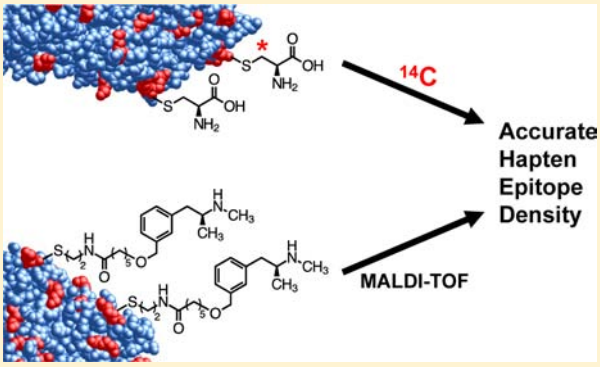
Supporting Information

ABSTRACT: Control of small molecule hapten epitope densities on antigenic carrier proteins is essential for development and testing of optimal conditions for vaccines. Yet, accurate determination of epitope density can be extremely difficult to accomplish, especially with the use of small haptens, large molecular weight carrier proteins, and limited amounts of protein. Here we report a simple radiometric method that uses ^{14}C-labeled cystine to measure hapten epitope densities during sulfhydryl conjugation of haptens to maleimide activated carrier proteins. The method was developed using a (+)-methamphetamine (METH)-like hapten with a sulfhydryl terminus, and two prototype maleimide activated carrier proteins, bovine serum albumin (BSA) and immunocyanin monomers of keyhole limpet hemocyanin. The method was validated by immunochemical analysis of the hapten–BSA conjugates, and least-squares linear regression analysis of epitope density values determined by the new radiometric method versus values determined by matrix-assisted laser desorption/ionization mass spectrometry. Results showed that radiometric epitope density values correlated extremely well with the mass spectrometrically derived values (r^2 = 0.98, y = 0.98x + 0.91). This convenient and simple method could be useful during several stages of vaccine development including the optimization and monitoring of conditions for hapten–protein conjugations, and choosing the most effective epitope densities for conjugate vaccines.



To generate high affinity antibodies against small molecular weight chemicals and peptides (e.g., <1000 Da molecular weight), multiple copies of a hapten must be covalently attached to a suitable antigenic carrier protein to form a vaccine. The degree of haptenation (or epitope density) can influence both the affinity and titer of the resulting antibody immune response.[1] However, the accurate measurement and optimization of epitope density is a critical and sometimes underappreciated aspect of vaccine development.

Two commonly used approaches for hapten–protein conjugation reactions are formation of a carboxamide[2] bond between the hapten and a reactive terminal amino group of the carrier protein, or a thioether bond between the hapten and a maleimide activated protein.[3,4] While the carboxamide chemistry is straightforward,[2] it is often difficult to achieve reproducible high levels of epitope densities on proteins (e.g., >10 per bovine serum albumin [BSA] molecule),[5] whereas with sulfhydryl chemistry it is relatively easy to achieve high epitope densities with maleimide activated proteins.[4]

In the process of developing an anti-methamphetamine (METH) vaccine, we realized the critical need to develop an inexpensive, rapid method for accurate quantitation of the epitope density on both small and large proteins. The colorimetric method of Ellman[6] is perhaps the most often used procedure for determining the number of sulfhydryl groups available for conjugation. However, the technique does not work well with all hapten–protein combinations or with very small-scale conjugation reactions where the amount of protein or antigenic carrier is a limiting factor. Chemical modification of the carrier proteins to allow direct conjugation of more haptens also adds to the complexity of the analysis. For smaller, commonly used carrier proteins (<100 kDa) such as ovalbumin, BSA, and thyroglobulin (TG), matrix-assisted laser

Received: October 3, 2014
Revised: November 25, 2014
Published: November 26, 2014

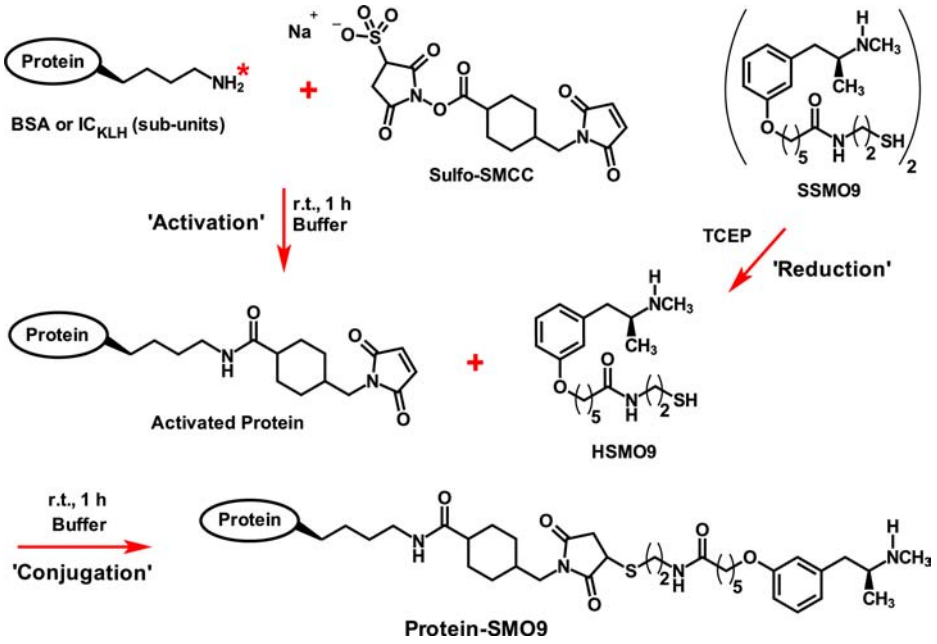


Figure 1. METH conjugate vaccine synthesis overview. The available lysine groups on a protein (BSA or IC_{KLH}) were "activated" with the cross-linker sulfo-SMCC in preparation for "conjugation"; for simplicity, only a single lysine terminal group is shown (*). Just prior to the conjugation step, TCEP is used as a reducing agent for the conversion of SSMO9 dimer to HSMO9 monomer. Starting with the dimer allows for just in time reduction of the highly reactive HSMO9 hapten. In the final step of the reaction, SMO9 is "conjugated" to the activated BSA or IC_{KLH} to form the METH-conjugate vaccine (labeled Protein-SMO9). After conjugation was complete, any unreacted maleimide groups of the BSA-SMO9 or IC_{KLH}-SMO9 vaccine were blocked or end-capped by the addition of a 4-fold excess of cysteine. This step was a precaution to prevent any potential chemical reactions of the vaccine with proteins in vivo.

desorption/ionization mass spectrometry (MALDI-TOF) is an accurate and reliable analytical method.[5,7−9]

Characterization of hapten−protein conjugates is especially difficult to achieve with large molecular weight proteins (e.g., >100 kDa) and very small haptens (e.g., <300 Da). For these larger carrier proteins and dimers (like IC_{KLH}) or multimers, accurate determination of mass can sometimes be best accomplished with radiolabeled haptens or with very advanced mass spectrometry systems. Both techniques are costly and not feasible for most laboratories.

We report here the utility and validation of a simple radiochemical method for accurate quantitation of sulfhydryl hapten conjugations to maleimide activated proteins. The method uses ^{14}C-cystine and the reduced form, ^{14}C-cysteine (^{14}C-Cys), as a tracer and maleimide activated carrier protein. To determine the feasibility of this analytical method we conjugated the METH-hapten SSMO9-METH [(*S*)-*N*-(2-(mercaptoethyl)-6-(3-(2-(methylamino)propyl)phenoxy)-hexanamide] to BSA (Figure 1) and used MALDI-TOF as a reference method to validate our results. We have previously published the synthesis of HSMO9,[4] and will publish the synthesis of SSMO9-METH in a future publication.

We also tested the method for use in the development of anti-METH vaccine using maleimide activated Immunocyanin (IC_{KLH}; Biosyn Corp., Carlsbad, CA) as the carrier protein (Figure 1). IC_{KLH} is a large molecular weight protein derived from the native Keyhole limpet hemocyanin (KLH; 8000 to 32 000 kDa), which consists of two stable subunit monomers with masses of ∼360 and ∼390 kDa, with abundant lysine residues for hapten linkage.[4,5,10,11] Relative to native KLH, IC_{KLH} is a more uniform antigenic carrier that is used in human vaccine clinical trials.[12,13] This IC_{KLH}-SMO9 conjugate vaccine is in preclinical development for the potential treatment of METH addiction.[4]

SSMO9 (Figure 1) is a new dimeric precursor of the METH-like hapten HSMO9 (hereafter referred to as the deprotonated SMO9), used for conjugation to IC_{KLH},[4] which is simpler to synthesize, does not require protection of the single SMO9 sulfhydryl group from degradation, and does not use mercuric acetate in the synthesis process. The elimination of possible mercury carryover in the reaction is a critical improvement for a potential human vaccine. In addition, the S−S dimer form of haptens can stabilize the labile SH group and protect it from unwanted side reactions during synthesis.[14] This can also improve the storage stability and quality of sulfhydryl-containing haptens. The reduced form of this hapten, SMO9, was directly conjugated to the maleimide activated BSA or IC_{KLH} (Figure 1). The activation, reduction, and conjugation reactions for the radiometric and MALDI-TOF analysis experiments as well as for vaccine production were performed similarly and are described in Supporting Information.

To characterize the maleimide activated proteins and confirm the conjugation of SMO9 to the activated protein, SMO9−activated BSA conjugates were evaluated by SDS-PAGE and Western blot analysis (Figure 2A and B). SMO9−activated IC_{KLH} conjugates were evaluated using an agarose gel system and Western blot (Figure 2C and D). Activation of both BSA (Figure 2A, lane 3) and IC_{KLH} (Figure 2C, lane 2) resulted in the formation of large molecular weight protein bands that did not react with an anti-METH antibody upon Western blot analysis (Figure 2B, lane 3 and Figure 2D, lane 2), but did react with the anti-METH antibody when the activated proteins were conjugated with SMO9 (Figure 2B, lanes 4−7 and Figure 2D, lane 3). This confirmed the coupling of the SMO9 hapten to the various forms of the activated proteins.

dx.doi.org/10.1021/bc500456z | *Bioconjugate Chem.* 2014, 25, 2112−2115

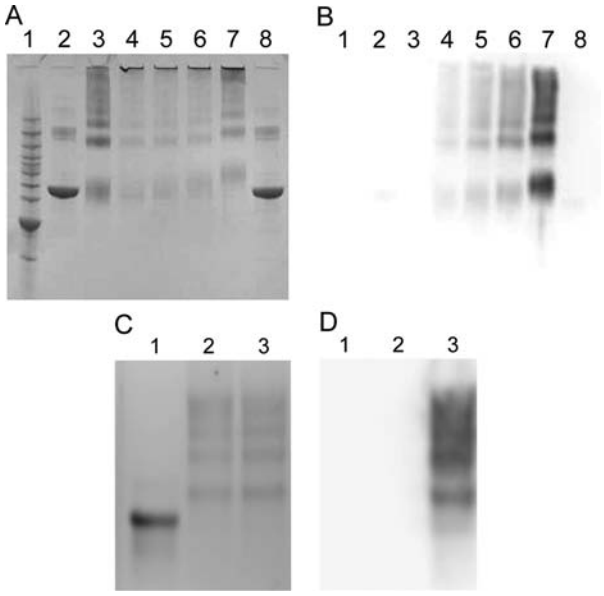


Figure 2. (A) SDS-PAGE showing BSA molecular weight increases as the ratio of SMO9 to BSA is increased. Lanes: (1) molecular weight markers; (2) unconjugated BSA at 1 mg/mL; (3) sulfo-SMCC activated BSA (1 mg/mL), (4–7) BSA:SMO9 ratios of 1:10, 1:15, 1:20, and 1:30, (8) unconjugated BSA at 1 mg/mL. (B) Western blot analysis of same lane order as 3A probed with anti-METH mAb4G9. Note the increase in image signal strength and in apparent molecular size as the ratio of BSA:SMO9 increases. (C) APE GEL analysis of IC_{KLH}-SMO9 conjugates stained with Coomassie. Lanes: (1) unconjugated IC_{KLH}; (2) maleimide activated IC_{KLH}; (3) IC_{KLH}:SMO9. (D) Western blot of same gel order as in C, probed with anti-METH mAb4G9.[2]

For the ^{14}C-Cys radiometric analyses of BSA–hapten conjugations, we determined both the incorporation of ^{14}C-Cys tracers in the presence of large amounts of SMO9 (SMO9-equivalents, a competitive reaction for protein conjugation sites), and the incorporation of the ^{14}C-Cys tracers in the presence of unlabeled cysteine (reported as ^{14}C-Cys-equivalents, a noncompetitive reaction). We hypothesized that parallel measurement of the incorporation of ^{14}C-Cys-equivalents, conducted at the same time as SMO9 protein conjugation reactions, would be the most accurate predictor of SMO9-protein epitope density. It was not clear if ^{14}C-Cys in the presence of large amounts of SMO9 would accurately predict epitope density, since the two molecules differed in structure and chemical properties. This could result in different rates for the completion of forming covalent bounds with the protein, and thereby produce an over or under estimation of the true SMO9 epitope density on the proteins.

To test this hypothesis we conducted three parallel conjugation reactions: (1) SMO9 with a ^{14}C-Cys tracer, (2) unlabeled cysteine with a ^{14}C-Cys tracer, and (3) SMO9 alone with a range of BSA:hapten ratios (1:10, 1:15, 1:20, and 1:30). The hapten epitope density of SMO9 alone (3) was determined by MALDI-TOF analysis, as described previously.[4] This was deemed our reference method for assay validation. The samples containing ^{14}C-Cys were quantitated by liquid scintillation spectrophotometry. Although the amount of the tracer radioligand bound to the protein was relatively low, we were able to use this incorporation of ^{14}C-Cys as a relative measure of the amount of conjugation of unlabeled cysteine or SMO9.

Since the values for coefficients of determination (r^2) were near 1.0 (Figure 3A), both the noncompetitive and competitive

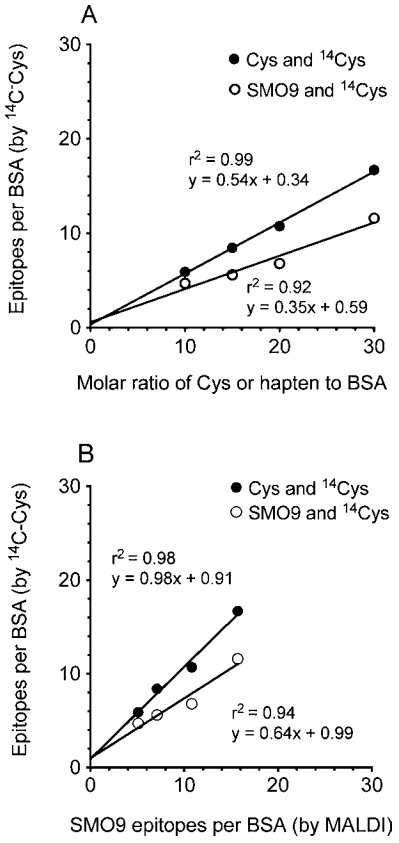


Figure 3. (A) Linear regression analysis of the number of epitopes incorporated in the radiometric assays (SMO9 with a ^{14}C-Cys tracer: open symbol; and unlabeled cysteine with a ^{14}C-Cys tracer: closed symbol) versus the ratio of SMO9 hapten to maleimide activated BSA. (B) Relationship between the predicted number of epitopes by the ^{14}C-Cys radiometric assays and the number of observed SMO9 epitopes by MALDI-TOF analysis. As judged by linear regression analysis, there was an excellent correlation between predicted and observed values in the cysteine and ^{14}C-Cys assay, but the SMO9 and ^{14}C-Cys analysis significantly underpredicted the true number of SMO9 epitopes.

radiometric assays showed a strong linear relationship throughout the range of BSA:hapten ratios. This indicated that the available maleimide groups did not reach saturation in either assay, but the differences in slopes between the noncompetitive and competitive radiometric assays (0.54 vs 0.35, respectively) suggested there was unequal competition for the covalent binding of ^{14}C-Cys and SMO9.

The epitope density values predicted from the two radiochemical assays in Figure 3A were then correlated with the direct MALDI-TOF measurements of SMO9 epitope density values using linear regression analysis (Figure 3B). Calculation of SMO9-equivalents based on the competitive SMO9-^{14}C-Cys tracer assay indicated that this method underestimated the SMO9 epitope densities compared to the MALDI-TOF analysis of SMO9 epitopes (slope = 0.64, r^2 = 0.94; Figure 3B). The measurement of epitope densities as ^{14}C-Cys-equivalents in the noncompetitive assay proved to be the accurate measurement of SMO9 hapten incorporation based on the excellent values for the slope, *y*-intercept, and coefficient of

dx.doi.org/10.1021/bc500456z | *Bioconjugate Chem.* 2014, 25, 2112–2115

determination (slope = 0.98, r^2 = 0.99; Figure 3B). Using this new method we also determined the average SMO9 hapten density on the IC_{KLH} dimers shown in Figure 2C and D was 14 haptens per IC_{KLH}.

These findings suggest that under these (noncompetitive) reaction conditions both ligands are able to achieve the same maximal binding, but perhaps at a different rate. This positive result was aided by the fact that we carefully optimized each of the reactions to allow maximal binding, which resulted in the same final epitope densities for each ligand.

This simple and versatile radiometric method for determining the extent of hapten−protein conjugation with sulfhydryl linkages could potentially be broadly useful in determining the epitope density of therapeutic vaccines (for treating drug abuse or other medical or veterinary purposes), antibody−drug conjugates, antibody−nanoparticle conjugate, and other protein conjugations. However, further testing and validation of the method with other hapten and protein combinations will ultimately determine the broader applicability and accuracy of the method.

Even if the analysis of a carrier protein conjugate is feasible by mass spectrometry or colorimetric assays, this relatively quick method has advantages because it is inexpensive compared to mass spectrometry, does not require radioactive haptens, can be used for measurements of very large and complex proteins (e.g., IC_{KLH} is a dimer), and, unlike protein colorimetric methods, only requires microgram quantities of protein. These advantages could be useful during the optimization and monitoring of conditions for hapten−protein conjugations, and choosing the most effective epitope densities for conjugate vaccines.

■ ASSOCIATED CONTENT

Supporting Information

Detailed methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): SMO has financial interests in and serves as Chief Scientific Officer of InterveXion Therapeutics LLC (Little Rock, AR), a pharmaceutical biotechnology company focused on treating human drug addiction with antibody-based therapy..

■ ACKNOWLEDGMENTS

The National Institute on Drug Abuse (U01 DA023900, R01 DA026423, T32 DA022981), Arkansas Biosciences Institute, and the National Center for Advancing Translational Sciences (UL1 TR000039) supported this work.

■ ABBREVIATIONS

BSA, bovine serum albumin; ^{14}C-Cys, carbon-14 labeled L-cysteine; IC_{KLH}, Immunocyanin monomers of Keyhole limpet hemocyanin; KLH, Keyhole limpet hemocyanin; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization Time of Flight; SMO9, mercapto-hapten (*S*)-*N*-(2-(mercaptoethyl)-6-(3-(2-(methylamino)propyl)phenoxy)hexanamide; METH, (+)-methamphetamine; Sulfo-SMCC, sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate; TCEP, Tris (2-carboxyethyl)phosphine

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