

Synthesis and Characterization of Hapten–Protein Conjugates for Antibody Production against Small Molecules

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For the generation of antibodies against small hapten molecules, the hapten is cross-linked with some carrier protein to make it immunogenic. However, the formation of such conjugates is not always reproducible. This may lead to inconsistent hapten–protein stoichiometries, resulting in large variations in the generation of the desired antibodies. In the study described here the hapten (mercaptopropionic acid derivative of atrazine) was coupled to carrier protein at five different molar ratios. The hapten–protein conjugates prepared were characterized thoroughly by spectrophotometric absorption, fluorescence, matrix-assisted laser desorption ionization (MALDI), and gel electrophoresis methods, before being used for the immunization and assay purposes. Electrophoresis and fluorescence methods were very useful in detecting hapten–protein cross-linking while MALDI-MS and spectrophotometric detection provided qualitatively comparable hapten density. The production of specific antibodies was sought following the generation of appropriate hapten–protein conjugates. A high antibody titer with moderate antibody specificity was obtained with hapten density around 15 molecules per carrier protein. The study proved useful for monitoring the course of hapten–protein conjugation for the production of specific antibodies against small molecules.

INTRODUCTION

Small molecules such as pesticides, drugs, etc. are usually nonimmunogenic and hence do not elicit an immune response unless coupled with some macromolecules such as proteins. It is, therefore, required to modify these small substances (hapten) for coupling with macromolecules (carrier) so as to make a stable carrier–hapten complex. **Synthesis of hapten for linking with carrier proteins is the most important aspect of specific antibody generation against small molecules for immunoassay applications (1–3).** The functional group of the hapten governs the selection of the conjugation method to be employed. Common procedures use amine, carboxylic acid, hydroxyl, or sulfhydryl groups on the hapten and the protein (4). The most frequently used carrier proteins for conjugation are bovine serum albumin (BSA), ovalbumin (OVA), conalbumin (CONA), thyroglobulin (TG), immunoglobulin (Ig), fibrinogen, or keyhole limpet hemocyanin (KLH). Hapten–protein conjugates made with serum albumin are more frequently used than conjugates of γ -globulin or other carrier proteins. The hapten–protein conjugates of bovine, rabbit, and human serum albumin were found to be soluble above pH 5.5. However, similar conjugates made of γ -globulin and egg albumin precipitated out of solution during preparation (5). **KLH, even though consisting of a large number of available lysines for hapten conjugation, its use as a carrier protein is restricted because of the problem of its solubility in various buffers due to its high molecular size.**

There has been significant progress in recent years in protein–hapten conjugates for the generation of anti-hapten antibodies with applications in immunoassays for environmentally hazardous pollutants (6–8). An improved method for the microscale preparation and char-

acterization of hapten–protein conjugates for nonchromophore hydroxylated haptens was demonstrated by Naar and co-workers (9). In another study, a solid-phase conjugation method utilizing carrier protein bound to an ion exchange matrix was developed, and the conjugation ratio was determined by acid hydrolysis and amino acid analysis (10). Verification of the coupling reaction and determination of the hapten density can be accomplished spectrophotometrically, mainly by evaluating the available free amino groups before and after conjugation (11, 12), by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) to determine the mass change before and after conjugation (13, 14) or by radiolabeled haptens (15). To prepare an effective hapten–protein conjugate for the desired immune response, it is important to characterize the resulting hapten–protein conjugates to determine the hapten density on carrier protein. The higher ratio of hapten usually increases the strength and specificity of the immune response. However, there is a risk that a high degree of substitution could adversely affect the activity and specificity of antibodies produced (16). Besides, the formations of protein–hapten conjugates are not always reproducible, even in best experimental conditions. This may lead to inconsistent **hapten–protein stoichiometries** resulting large variation in desired antibodies generation. Careful selection of hapten density for hapten–protein conjugation is therefore an important aspect of antibody generation. In the present study, the binding efficiency of hapten–carrier conjugate (number of hapten molecules per protein molecule) was optimized by taking different hapten: carrier molar ratios. Mercaptopropionic acid derivative of atrazine (MPAD) was synthesized in our laboratory for coupling with carrier protein. Five molar ratios of hapten were used to make hapten–protein conjugates for immunization purpose. The optimum molar ratio of hapten to protein to produce conjugates

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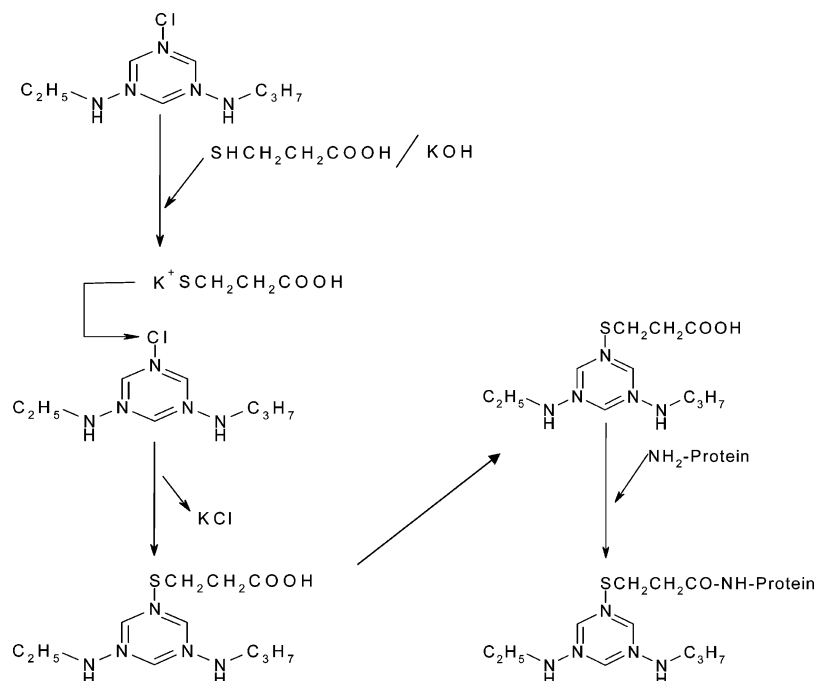


Figure 1. Synthesis and conjugation of MPAD derivative of atrazine with protein.

for immunization was determined according to a balance between good antibody titers and specific antibodies generated.

MATERIALS AND METHODS

Reagents. Bovine serum albumin (BSA), dicyclohexyl carbodiimide (DCC), *N*-hydroxysuccinimide (NHS) ester, 3-mercaptopropionic acid, 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS), fluorescein isothiocyanate (isomer 1), peroxidase conjugated goat anti-rabbit immunoglobulin, normal anti-rabbit immunoglobulin, and 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid) were purchased from Sigma Chemical Co., St. Louis, MO. Technical grade atrazine was purchased from Supelco. Peroxidase (POD) and 3,3',5,5'-tetramethylbenzidine (TMB) substrate were obtained from Bangalore-Genei, India. Affi-gel-102 was purchased from Bio-Rad. All chemicals, reagents, and solvents used in this study were of high purity analytical grade. Buffers were made in Milli-Q double distilled water.

Synthesis of Atrazine Derivative. Mercaptopropionic acid derivative of atrazine (MPAD) was synthesized in our laboratory by the method described by Goodrow and co-workers (2). Briefly, in a solution of 5.01 mmol of atrazine (Tech. Grade) in 50 mL of ethanol was added slowly, under constant stirring, a solution of 3-mercaptopropionic acid (5.5 mmol) and 85% KOH (10.8 mmol) in 10 mL of ethanol. The mixture was refluxed for 6 h, and then the solvent was evaporated under reduced pressure. The residue was taken up in 25 mL of 5% NaHCO_3 and washed three times with 10 mL of chloroform. The aqueous layer of the solution was acidified (pH 2.0) with 6 N HCl, causing the acidic derivative to precipitate immediately. The supernatant was decanted, and the derivative was dried under mild vacuum. The precipitate was further dissolved in 1 mL of ethanol and then allowed under reduced pressure at 37 °C to form MPAD crystals. The product was stored in a tightly sealed bottle for further use.

Conjugation of Hapten with Protein. Conjugates of atrazine (MPAD derivative) with protein (BSA) at five different molar ratios of protein-hapten (1:5, 1:10, 1:20,

Table 1. Different Molar Ratios of Protein-Hapten Used for Making Conjugates M1 to M5

protein concentration	hapten concentration	protein-hapten ratio
0.15 μmol (10 mg)	0.75 (15 μL)	1:5 (M1)
0.15 μmol (10 mg)	1.50 (30 μL)	1:10 (M2)
0.15 μmol (10 mg)	3.00 (60 μL)	1:20 (M3)
0.15 μmol (10 mg)	6.00 (120 μL)	1:40 (M4)
0.15 μmol (10 mg)	15.0 (300 μL)	1:100 (M5)

1:40, and 1:100, M1-M5) were prepared. The stock solution of protein (10 mg/mL; 0.15 μmol) was made in pH 9.0 borate buffer, and the final reaction volume of the protein-hapten conjugates was kept constant at 1 mL for each preparation. Stock solution of haptens was made by adding 50 μmol of MPAD derivative of atrazine in 1 mL of DMF along with 125 μmol of DCC and 125 μmol of NHS. The mixture was incubated for 4 h at RT and then centrifuged for 5 min at 10 000 rpm to remove the urea precipitate. The reaction mechanism of MPAD-protein conjugation method is shown in Figure 1. The supernatant was used to prepare conjugates with protein by adding different amounts of hapten to a fixed amount of protein (10 mg) in a final volume of 1 mL to make protein-hapten conjugates of different molar ratios as shown in Table 1. The conjugates were passed through P10 gel filtration column (Pharmacia, Sweden). The fractions with maximum protein concentration were collected, pooled, and checked for the final concentration of protein (hapten-protein) with UV spectrophotometer at 280 nm.

Characterization of Conjugates. Protein-hapten conjugates were characterized by following physical techniques to determine the extent of conjugation.

(a) Spectrophotometric Analysis. The available groups of surface lysine present in carrier proteins before and after conjugation (made at different molar ratios of hapten-protein) were determined using 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) reagent. The amount of amino groups present in the carrier protein before and after coupling with carboxylated hapten was directly quantitated with a UV/vis spectrophotometer (Shimadzu 1601) at 335 nm. Different conjugates were prepared at

Table 2. Determination of Hapten Density on BSA–MPAD Conjugates Using Chemical TNBS and Physical MALDI–TOFF Mass Spectrometric Method^a

conjugate	carrier–hapten molar ratio used for preparation of conjugates	chemical TNBS method		mass spectrometry MALDI–TOFF		$\Delta M/M_h$ (hapten density ^c)
		% amino groups consumed	approximate no. of amino groups used	observed mass ^b (kDa)	change in mass (ΔM)	
control	1:0	0.0	0.0 (0)	67221.0	0	0
M1	1:5	6.0	3.48 (3)	67744.9	523.9	1.84 (2)
M2	1:10	8.0	4.64 (5)	68293.8	1072.8	3.76 (4)
M3	1:20	18.0	10.44 (10)	70288.7	3067.7	10.75 (11)
M4	1:40	24.0	13.92 (14)	71720.8	4499.8	15.77 (16)
M5	1:100	29.0	16.82 (17)	<i>d</i>	—	—

^a The number of lysine groups on protein being utilized for conjugates M1 to M5 before and after conjugation are given in brackets.

^b Mass of hapten (MPAD) $M_h = 285.4$. ^c Number of hapten molecules per BSA molecule. ^d Did not fly.

a concentration of 1 mg/mL and were reacted with 0.1% TNBS solution under alkaline conditions to determine the percentage of NH_2 groups used during conjugation in different conjugates. An amount of 200 μL of conjugate solution was taken and mixed with 200 μL of 4% NaHCO_3 solution. An amount of 200 μL of 0.1% TNBS solution was added to the mixture and incubated for 1 h at 37 °C. The color of the solution was read at 335 nm. The amount of NH_2 groups used during conjugation of pesticide residues to the BSA molecules was determined from the difference between the O.D. of the control and the conjugate.

(b) Fluorescence Spectra. The emission spectra of hapten–protein conjugates (M1–M5) were taken in the range of 300–450 nm after excitation at 290 nm (tryptophan excitation) using Kontron spectrofluorometer (SFM 25). The conjugates were also excited at 270 nm to see the effects of both tyrosine and tryptophan amino acids on the conjugates. Spectra were obtained using 1 nm excitation and emission slits, and were recorded at a scan rate of 5 nm s^{-1} .

(c) Gel Electrophoresis. The native and SDS–PAGE of the conjugates (different hapten–protein molar ratio) was performed using Bio-Rad electrophoresis apparatus according to method developed by Laemmli (17). Solutions of the conjugates were prepared in SDS–PAGE sample buffer (10 mM Tris, 1 mM EDTA, 2.5% SDS, and 5% mercaptoethanol, pH 8.0) to 1 mg/mL and heated to 100 °C for 5 min. Gels were loaded with 5 μL of sample and run under denaturing conditions for 90 min at 120 V. Coomassie blue staining was done using a standard developing protocol supplied by the manufacturer. The gel was washed three times in Milli-Q water, followed by staining for 1 h with stain reagent followed by washing in Milli-Q water.

(d) MALDI-TOF. The mass analysis of hapten–protein conjugates was done with MALDI spectrometer (Kratos Analytical Systems). Sinapinic acid solution was prepared at a concentration of 15 mg/mL in acetonitrile. Protein conjugates dialyzed against distilled water were used at a concentration of 1.5 mg/mL. The conjugate samples and matrix solution were mixed in equal amounts (1 μL each) and added on the stainless steel probe having 0.5 μL of TFA solution (0.1%). The samples were allowed to dry at room temperature and then kept in the system for MASS analysis. The data were acquired with 50 shots per sample in the linear mode at 30 kV and analyzed using the software provided with the system.

Antibody Generation. The hapten–protein conjugates were used to immunize mice and rabbits subcutaneously by taking 25 μg and 250 μg of each conjugate, respectively, emulsified with Freund's complete adjuvant. This was followed by three secondary boosters of same dose with incomplete Freund adjuvant at intervals of 21 days. The mice and rabbits were bled on the 5th day after

the each boost, and the antibody titer was determined. After the confirmation of good titers (checked by ELISA), sufficient amount of sera were prepared and the immunoglobulin fraction was purified using a protein A-sepharose column (Amersham-Pharmacia Biotech). The relative functional affinity of the antibodies was measured by diethylamine dependent displacement of antigen–antibody interaction. Results were expressed as the logarithmic increase in antibody concentration required to obtain the same level of O.D. at 50% of saturation in the absence of diethylamine (18).

ELISA Procedure. ELISA plates (96-well polystyrene from Imperial Biomed, India) were coated with 100 μL of each conjugate (25 $\mu\text{g}/\text{mL}$) made in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After being coated, the plates were washed thoroughly with phosphate buffer saline (50 mM, pH: 7.2). Unbound sites of the plates were blocked with 5% defatted skim milk in PBS for 2 h at 37 °C. The plates were washed three times with PBS containing 0.05% Tween-20 (PBST) and finally with PBS. Primary antibodies (serum) incubation was done for 3 h at 37 °C by adding 100 μL of antibody solution in each well at a dilution of 1:1000 in PBS. After incubation, the plates were thoroughly washed three times with PBST and finally with PBS. The plates were then incubated with 100 μL of HRP conjugated goat anti-mouse immunoglobulin (1:5000 dilution) for 1 h at 37 °C. The plates were washed as before, and the color was developed by adding 100 μL of TMB/ H_2O_2 solution. The reaction was stopped with 50 μL of 1 N H_2SO_4 . Optical density at 450 nm was measured using an ELISA plate reader (Molecular Devices).

RESULTS

Analysis of Conjugates. TNBS Method. Covalent attachment of carboxylic acid haptens to protein (BSA) via available surface amino groups on BSA (26 surface L-lysine) was confirmed by reacting all the prepared conjugates (M1–M5) with TNBS reagent. The number of amino groups present in the carrier protein before and after conjugation was quantitated with UV/vis spectrophotometer (Shimadzu 1601) at 335 nm and shown in Table 2. The gradual decrease in the available surface lysine on protein after reaction with different molar ratios of hapten confirms the increase in conjugation density (number of hapten molecules per protein molecule) with increasing hapten–protein molar ratio.

Fluorescence Method. The fluorescence emission spectra for each hapten–protein conjugate in the range of 300–450 nm after excitation at 290 nm (excitation of tryptophan residues only) are shown in Figure 2. The combined effects of both tyrosine and tryptophan amino acids in conjugates were also checked by exciting the samples at 270 nm and monitoring the spectra between

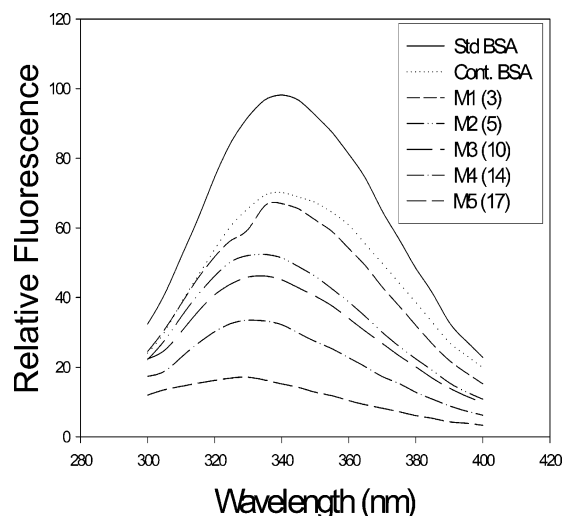


Figure 2. The fluorescence emission spectra (λ_{ex} at 290 nm) of hapten-protein conjugates M1, M2, M3, M4, and M5 having average hapten densities of 3, 5, 10, 14, and 17, respectively.

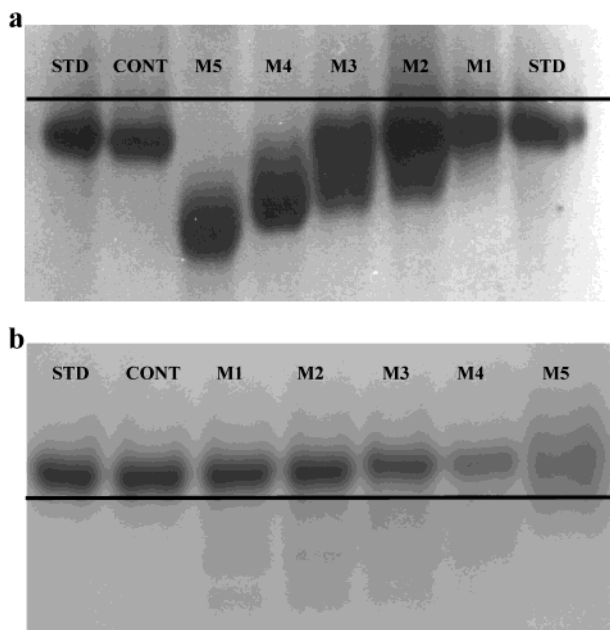


Figure 3. Gel electrophoresis of conjugates (a) native gel and (b) SDS-PAGE. Different BSA-atrazine conjugates M1, M2, M3, M4, and M5 were prepared by reacting activated hapten (MPAD) in molar excess of 1:5, 1:10, 1:20, 1:40, and 1:100, respectively.

300 and 450 nm. No significant change in fluorescent intensity was observed at 270 and 290 nm excitation for the hapten conjugated with protein. The fluorescence spectrum analysis was therefore performed at 290 nm excitation wavelength. Conjugates with increasing molar ratios of protein-hapten (M1-M5) showed linear decrease in fluorescence intensity. The fluorescence intensity of M5 conjugate having protein-hapten molar ratio 1:100 showed almost zero fluorescence intensity.

Gel Electrophoresis and MALDI-MS Analysis. SDS-PAGE and native gel electrophoresis of conjugates are shown in Figure 3. The use of pure protein (BSA) and conjugates made by using different molar ratio of hapten as generated by native gel showed well-defined bands with gradual increments for each hapten with increase in molar ratio (Figure 3a). For the SDS-PAGE (Figure 3b), it was observed that the BSA molecules with higher conjugation density moved further, as this con-

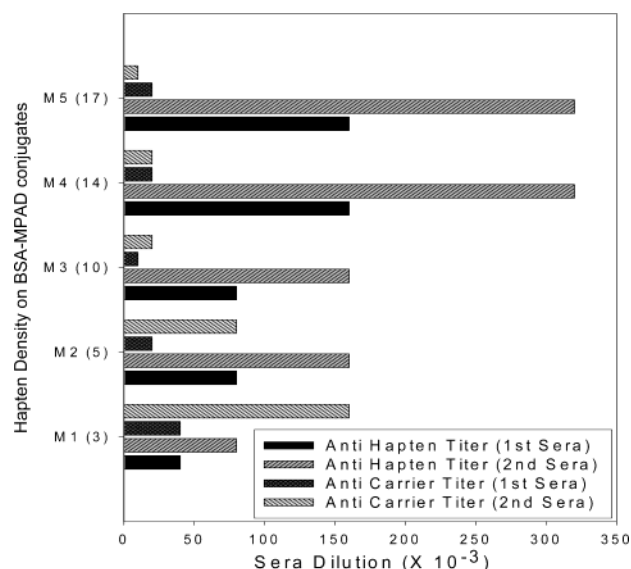


Figure 4. The titer of anti-hapten (atrazine) and anti-carrier (BSA) antibodies in antisera. Rabbits were immunized with various BSA-atrazine conjugates (M1 to M5) having average hapten densities of 3, 5, 10, 14, and 17, respectively.

jugate is more negatively charged in comparison to conjugates prepared with low molar ratio. The SDS-PAGE results correspond to the mass pattern obtained by MALDI-TOF spectrometer. Conjugation density for each hapten in increasing molar ratio (M1-M5) resulted in a detectable increase in the molecular weight of the conjugate as determined by observing the peak shift of mass spectrum (Table 2). The molecular weight of each conjugate was calculated from the peak centroid of the peaks. The incremental change in molecular weight due to incorporation of hapten molecules to protein corresponds to the number of hapten molecules per protein molecule. Apparent coupling efficiency for the MPAD to BSA, at different molar ratios, was nearly same.

ELISA Results. All conjugates were tested for their immunogenicity by checking the antibody titers in serum after immunization. The titer of the serum was defined as the dilution giving 50% maximal response by ELISA. Immunization of mice with conjugates (M1 to M5) produced different amount of antibody titers when tested by ELISA (Figure 4). A relatively good titer of anti-hapten antibodies was obtained with M4 conjugate (hapten: protein ratio 1:40). The further increase in conjugational density, i.e., conjugates with hapten-protein ratio of 1:100 (M5), did not show any increase in specific anti-hapten antibodies.

Antibody Characterization. To characterize the antibodies generated against different conjugates, ELISA was done to determine the sensitivity of generated antibodies. The antibody obtained against M4 conjugate, as determined by standard dilution curve analysis, showed very good sensitivity up to the level of ng/mL (Figure 5).

DISCUSSION

For the generation of specific antibodies against small molecules, these molecules are synthesized and conjugated with carrier proteins in such a way that they mimic the structure of the compound and contain a reactive group that can form a covalent linkage with the carrier proteins. The functional group of the hapten governs the selection of the conjugation method to be employed. The method used in this study utilized carbodiimide method

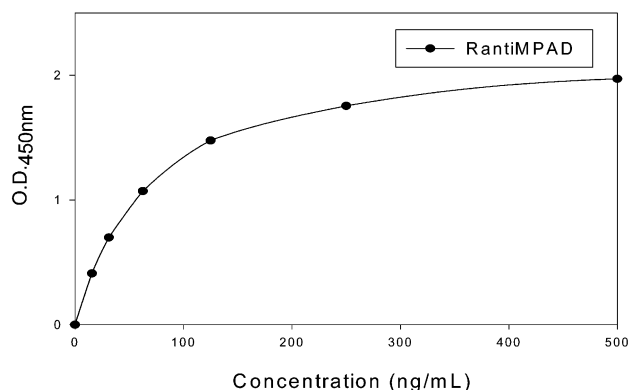


Figure 5. Standard dilution curve for antibody obtained against conjugate M4. Antibody was tested for its sensitivity by dilution curve analysis of data obtained between 12.5 ng/mL to 500 ng/mL of antibody concentration.

(19) for carboxy acid haptens (atrazine derivative) to link with BSA. This approach ensured stable cross-linking of haptens with protein along with *N*-acylurea formation. The optimum use of high grade of reagents along with thorough dialysis to remove unbound hapten and *N*-acylurea resulted in good coupling efficiency of haptens to protein as shown by SDS-PAGE and native gel electrophoresis results (Figure 3a,b). Each conjugate generated well-defined bands with low diffusibility. In native gel analysis, the gradual movement of bands with an increase in hapten density is due to a change in net charge of the conjugates, since for each lysine on protein utilized for the conjugation with hapten, one positive charge is lost. Thus, conjugates with more haptens per protein become relatively more negative and move farther than native BSA. Although the electrophoresis analyses of conjugation could not give definite information about the degree of hapten density, its main advantage was in detecting the course of cross-linked conjugation (21). In MALDI analysis, the relative increase in the molecular weight of the conjugates is apparent by gradual mass peak shift (Table 2). This corresponds to an increase in hapten density of conjugates, thus determining accurately the number of haptens per protein molecule. A BSA molecule with molecular weight of 66 531 Da (from the manufacturer's data sheet) showed a gradual shift in mass peaks with conjugates made with different molar ratios of hapten (MPAD derivative of atrazine). BSA with 26 available surface lysines can theoretically bind up to 26 hapten molecules. However, the estimated number of hapten molecules linked to BSA could not be more than 17, even at the highest hapten density, i.e., hapten-protein ratio of 1:100. The reason for a lower binding limit of haptens to the protein might be the protein conformational changes induced by haptens. This has also been observed in our analysis of fluorescence spectra for different conjugates (Figure 2) where tryptophan intensity of the hapten-protein conjugates shift toward the blue region for conjugates with higher hapten density, respectively. Due to this compactness of the protein, some surface lysine might actually be in the buried region of the protein, thus allowing fewer lysine groups for linking with the hapten.

The fluorescence spectra for different hapten-protein conjugates showed a linear decrease in fluorescence intensity with increase in hapten-protein molar ratio. The fluorescence signal at 290 nm excitation and 340 nm emission is mainly due to a number of tryptophan residues being available in the protein. The decrease in fluorescence intensity at this wavelength is due to

quenching of tryptophan intensity with increase in hapten density, i.e., increase in the number of amide bonds formed between the surface lysine groups of the protein and carboxylated hapten (atrazine derivative). This gradual shift in the fluorescence signal or quenching of tryptophan intensity with different conjugates thus confirms the course of hapten-protein conjugation.

The hapten density of the conjugate is an important parameter which generally defines the quality and quantity of antibody produced. A good antibody titer with higher specificity could be generated with an optimum number of haptens per protein molecule. Higher substitution resulted in an IgM response exceeding that of IgG and produced antibodies of lower affinity. Similar observations have been reported by some other groups (20–21). It is assumed that high antibody titers with moderate antibody affinities are usually obtained with hapten density of 15–30 molecules per carrier protein while a lower hapten density induces a slower immune response with higher affinity antibodies.

In conclusion, we have characterized different conjugates for getting an optimum molar ratio of protein-hapten conjugate for immunization purposes. The spectrophotometric method using trinitrobenzenesulfonic acid (TNBS) and MALDI-MS were able to quantify the hapten-protein conjugation density accurately. A new fluorescence method has been developed to confirm the course of conjugation by monitoring the relative shift in fluorescence intensity of conjugates. However, the role of many parameters, such as hapten polarity, type of solvent, and the nature of quenching, on the fluorescence signal have to be studied before this method can be used for quantitative determination of conjugation efficiency. This work is undergoing in our laboratory.

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