Brief Report

DOI: 10.5582/bst.2012.v6.2.52

Influence of hapten density on immunogenicity for anti-ciprofloxacin antibody production in mice

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Summary **To generate antibodies against small molecules, it is necessary to couple them as haptens to large carriers such as proteins. However, the immunogenicity of the conjugates usually has no linear correlation with the hapten-protein ratio, which may lead to large variations in the character of the desired antibodies. In the present study, ciprofloxacin (CPFX) was coupled to bovine serum albumin (BSA) in five different proportions using a modified carbodiimide method. The conjugates were characterized qualitatively by spectrophotometric absorption and electrophoresis methods. Mass spectrometry and the trinitrobenzene sulfonic acid method were adopted to assay the density of conjugates quantitatively. As a result, CPFX-BSA conjugates with various hapten densities (21-30 molecules per carrier protein) were obtained. After immunization in mice, ELISA tests showed that the antisera titer increased gradually with the increase of hapten density. The antibody obtained from the mice showed high sensitivity toward CPFX. These results revealed the relationship between hapten density and immunogenicity as well as an optimized conjugation approach for immunization purposes.**

Keywords: Hapten density, conjugation, immunogenicity, ciprofloxacin

1. Introduction

A small hapten (molecular weight < 1,000) is usually not immunogenic by itself (*1,2*). It is well-known that immunogenicity can be acquired when a hapten is coupled with a macromolecule carrier, such as a protein, peptide or synthetic amino acid (*3*). A hapten is generally coupled to a carrier protein through the ε-amino group. The carrier protein can increase both the strength and specificity of the antibody response efficiently. The coupling ratio of the hapten-protein is usually important for the properties of the antibody induced by the modified hapten. In most cases, increasing of the coupling ratio can enhance the strength and specificity of the immune responses (*4*). On the other hand, the higher coupling ratio may decrease the activity of antibodies. The possible relationship between the immunogenicity and the hapten-protein ratio may cause large variations in the character of the desired antibodies. Screening an optimal hapten density for the conjugation is significant to improve the binding efficiency of hapten-protein conjugation (*5*).

Ciprofloxacin (CPFX) is an antibacterial agent applied as a veterinary medicine. The CPFX residues in consumed animal tissues raise potential risks for development of drug-resistance and chronic adverse effects in public health (*6*). The maximum residue limits (MRLs) of CPFX in food stuffs of animal origin were established by the European Union (*7*). Antibody-antigen reactions were extensively used to detect and quantify the CPFX residue in biological fluids. Such immunoassays were developed for determination of protein antigens as well as small molecule haptens. The central problem of fast detection by immunoassays was the availability of a specific antibody with high titer.

Although there are reports about CPFX conjugation (*1,8*), the varying hapten density leads to the unstable character of the antibodies to a great extent. Hence, the optimized coupling ratio of CPFX-bovine serum

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albumin (BSA) has still not been attained. In our previous studies, a monoclonal antibody (mAb) against small hapten-CPFX with high affinity and specificity was produced and was used for rapid CPFX immunoassays in food stuff of animal origin (*9*). Furthermore, with the CPFX-specific mAbs, an indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed for the sensitive and specific detection of CPFX residues in fishery products (*10*).

In this study, CPFX as a hapten was covalently attached to BSA by a modified carbodiimide method using 1-ethl-3-carbodiimide methiodide (EDC) (*1,8*). To generate the anti-CPFX antibody, the hapten density was optimized to improve the binding efficiency. It could be a benefit to the approach for preparing antibodies with good titre and sensitivity, which was significant for the CPFX immunoassay.

2. Materials and Methods

2.1. *Chemicals and animals*

CPFX (content ≥ 98.5%) was purchased from Zhejiang Guobang Pharmaceutical Co., Ltd. (Shangyu, Zhejiang, China). BSA was obtained from Dingguo Biotechnology Co., Ltd. (Beijing, China). Trinitrobenzene sulfonic acid (TNBS) was from Sigma-Aldrich (St Louis, MO, USA). EDC (purity ≥ 99.3%) was obtained from Yanchang Confident Biochemical Technology Co., Ltd. (Shanghai, China). Chemical reagents such as NaCl and K_2HPO_4 were from Guoyao Chemical Reagent Co., Ltd. (Shanghai, China). All the chemical reagents and solutions used in this paper were analytical grade. The buffer was prepared with double distilled water.

Individual Balb/c mice were purchased from the Second Military Medical University (Shanghai, China). All mice were housed under controlled conditions and received food and water *ad libitum*. All animal experiments were performed in accordance with the guidelines of Regulation on Animal Experimentation and were approved by State Scientific and Technological Commission, State Council, China.

2.2. *Coupling of the CPFX hapten and carrier protein*

The conjugates of BSA and CPFX were synthesized by a modified carbodiimide method using EDC (*11*). The conjugation reaction was performed with five different molecular ratios of BSA and CPFX (1:160, 1:320, 1:480, 1:640, and 1:800). CPFX was mixed with BSA (2 mg/mL) and EDC (60 mg/mL) while the reaction was carried out in phosphate buffer solution (pH 5.0) and incubated at 28°C for 2 h. The mixture was dialyzed against the same buffer for 2 days and then freeze-dried. The CPFX-BSA conjugates C1, C2, C3, C4, and C5 (see Table 1 for the molecular ratios) obtained were stored at –20°C until use.

2.3. *Spectrophotometric analysis*

The numbers of free Lys residue ε-amino groups in BSA conjugates were determined by the TNBS method (*12*). Conjugate samples (0.2 mg each) were dissolved in 1 mL of 0.1 M sodium carbonate solution and then 0.5 mL of 0.01% TNBS solution was added. After incubation for 2 h at 37°C, 0.5 mL of 10% sodium dodecyl sulfate (SDS) solution and 0.25 mL of 1 M hydrochloric acid solution were added to terminate the reaction. The absorbance was measured at 335 nm. The number of amino groups left on the BSA molecule after the coupling reaction was determined by the difference of optical density (OD) values between the control group and the coupling group.

CPFX-BSA conjugates (C1-C5) were scanned from 250 nm to 350 nm using a Spectronic UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Madison, WI, USA). The resolution length range and scanning speed were 1 nm and 1 nm/sec, respectively.

2.4. *Gel electrophoresis analysis*

Apparent molecular size of CPFX-BSA conjugates was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a PowerPac 300 type electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The conjugates (C1-C5) were dissolved in sample buffer (10 mM Tris, 1 mM EDTA, 2.5% SDS, and 5% mercaptoethanol, pH 8.0) with a final concentration of 1 mg/mL. After heating at 100°C for 5 min, 10 μL of each sample was loaded. The samples were separated at 80 V in the stacking gel and 100 V in a 12% separating gel. The protein samples were stained for 4 h using the Coomassie Brilliant Blue staining method and then de-stained by de-staining solution until the background was transparent.

2.5. *Mass spectrometry analysis*

The Agilent 1100 hp LCQ DECA Liquid Chromatography Mass Spectrum (Agilent Technologies, Santa Clara, CA, USA) was used to determine the molecular weights of the conjugates. The conjugates were dissolved in methanol and 10 μL of each was collected for injection. The chromatographic conditions were as follows. For gradient elution, ratios of mobile phases A and B (phase A, 20 mM sodium acetate + 0.018% triethylamine + 0.3% tetrahydrofuran, pH 7.2; phases B, 100 mM sodium acetate/acetonitrile/methyl alcohol (20:40:40, v/v/v, pH 7.2)) were 95:5 (v/v) during 0-5 min and gradually changed to 5:95 (v/v) during 5-17 min. The chromatography was performed at 25°C with a flow rate of 0.2 mL/min using a Zorbax 300SB-C18 column (Agilent Technologies). The workstation was operated in positive-ion linear mode with the following parameters:

Table 1. Determination of hapten density of CPFX-BSA conjugates by chemical TNBS method and mass spectrometry analysis

Conjugates	BSA-CPFX mole ratios in the reaction mixture	Chemical TNBS method		Mass spectrometry analysis		ΔM/Mh[a] (hapten density)
		Observed amount of amino group consumed (%)	Calculated amount of amino group consumed (moles/BSA molecule)	Observed molecular mass (Da)	Mass variation (ΔM)	
Control	1:0	0	0 (0)	66,210	0	0 (0)
C1	1:160	36.2	21.7 (22)[b]	73,015	6,805	20.6 (21)[b]
C2	1:320	41.5	24.9 (25)	74,202	7,992	24.1 (25)
C3	1:480	41.5	24.9 (25)	74,622	8,412	25.4 (26)
C4	1:640	47.1	28.2 (28)	75,216	9,006	27.2 (28)
C5	1:800	49.9	29.9 (30)	76,100	9,890	29.9 (30)

[a] Mh indicates molecular weight of CPFX hapten, Mh = 331.1; [b] Values in parentheses indicate deduced moles of CPFX binding on each BSA molecule.

20 kV accelerating voltages, 100 nsec extraction delay, and 500 m/z low mass gate (*13*). The molecular weight of the sample was analyzed with Data Explore™ software (Applied Biosystems, Carlsbad, CA, USA).

2.6. *Immunization*

The female 4 weeks old Balb/c mice were kept in the sterile room with feeding every day. CPFX-BSA conjugates (150 μg each) and Freund's complete adjuvants were mixed in the ratio of 1:1 to immunize the Balb/c mice. The antigen sample with adjuvant was intraperitoneally injected into the mice and boosted after two weeks. One week later, the mice were immunized again in the same way without adjuvant. After 10 days, a part of the blood sample was collected to determine the antisera titer by ELISA, and then 150 μg each of CPFX-BSA conjugate was injected into the mice by tail intravenous injection. Three days later, the blood sample of the mice was collected and kept at 4°C overnight. The sample was centrifuged at 7,000 rpm for 10 min. The supernatant was collected to determine the titer and lyophilized.

2.7. *ELISA*

A hundred μL of CPFX-conjugated ovalbumin, which was prepared in our laboratory (*10*), with a concentration of 400 ng/mL was added into wells of an ELISA plate (Dingguo Biotechnology Co., Ltd.) and incubated at 37°C for 1 h. After coating, the wells were washed 3 times (3 min each) with phosphate buffer containing 0.5% Tween-20 (washing solution). The wells were blocked with 200 μL 5% milk and stored at 4°C overnight. Two additional wells were filled with 100 μL buffer to serve as a control for nonspecific binding. After blocking, 100 μL of immunized mice sera (antisera) were added to the wells and incubated for 1 h at 37°C. After rinsing with washing solution, 100 μL of goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) (1:6,000) was added and incubated for 0.5 h at 37°C. After washing 3 times, color development was initiated by adding 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB)/H_2O_2 and, 10 min later, stopped by adding 50 μL of 2 M H_2SO_4. The absorbance was determined at 450 nm with a BioTek EL311 micro-plate reader (BioTek, Winooski, VT, USA).

2.8. *Detection of the sensitivity of antisera by indirect competitive ELISA*

The procedure of indirect competitive ELISA was similar to the ELISA process with some modifications. After blocking, 50 μL of suitably diluted antisera was added to each well and 50 μL samples of various concentrations of CPFX solution (2, 4, 6, 8, and 10 ng/mL) were added. The subsequent steps were the same as described above.

3. Results and Discussion

CPFX-BSA conjugates with various hapten densities were synthesized as described in Materials and Methods. In UV scanning spectra (250-350 nm), the maximum absorption peaks of BSA, CPFX, and CPFX-BSA conjugates were at 280, 270, and 275 nm, respectively, and the higher binding molar ratios of the carrier hapten-protein (C1-C5) correlated with stronger UV absorbance (data not shown). SDS-PAGE analysis of the conjugates (C1-C5) revealed that the molecular weights of the conjugates were larger than that of BSA (data not shown). These results suggest the success of the coupling reaction between CPFX and BSA.

The amount of free amino acid residues on the BSA molecule before and after coupling reaction was determined using the TNBS method and the amount of CPFX binding to the carrier BSA was calculated. With mass spectrometry analysis, the density of the hapten bound to the carrier protein was also determined by comparing the variations of molecular weight. As shown in Table 1, hapten densities of the conjugates increased gradually with increase of the hapten-protein molar ratios.

Next, mice were immunized with the conjugates C1-C5 and the antisera titer was determined by ELISA

as described in Materials and Methods. As shown in Figure 1A, the titer rose according to the increase of the ratio between carrier protein and hapten and reached 5.76×10^5 at the ratio of 1:800 (C5). In contrast, the antisera titer with different hapten density did not show a significant difference (data not shown). The sensitivity of the antibody produced by the coupling reaction was determined by the ELISA method. The standard dilution curve analysis of the antibody prepared by the conjugate C5 exhibited good sensitivity up to a level of ng/mL (Figure 1B). By indirect competitive ELISA, the IC_{50} value was 10.7 ng/mL (data not shown).

To acquire immunogenicity, the hapten needs to be coupled with a macromolecule (such as a carrier protein) and become a complete antigen. Generally, small molecules can covalently bind to a carrier protein. Different coupling methods are chosen and designed based on the different functional groups of the hapten. By the traditional EDC method, the conjugation efficiency for CPFX is not high enough as expected and the max value of hapten densities can reach 16 (*1*). In the traditional method, NHS is used as the activating group and can promote the carboxyl group to couple with the amino group (*1,8*), while the modified one without NHS can lead to a higher coupling efficiency. Unlike other fluoroquinolones, CPFX is abundant in amino and carboxyl groups. To couple CPFX, NHS may not be necessary for this reaction. In this method, the extra hapten and EDC were removed by extensive dialysis to ensure the accuracy of the following analysis. BSA is widely used as a carrier protein, because it contains various amino residues on the surface. Compared to tyrosine, tryptophan and imidazole residues, the lysine residue epsilon amino group allows CPFX hapten to couple much more easily by a covalent bond (*1*).

The production of the conjugates can be confirmed by SDS-PAGE. The greater amount of protein molecule bound by the hapten and the larger molecular weight of the conjugate correspond with the shorter migration distance. Electrophoresis analysis can analyze the process of conjugate coupling. Compared with the molecular weight of BSA (66 kDa) (*14*), the differences between different conjugates are very slight (3 kDa) (data not shown). As a result, the differences between the migration distances of the conjugates (C1-C5) were not significant. The hapten density of the conjugates can be calculated more precisely by comparing the molecular weight variations of the conjugates with mass spectrometry. There are 59 lysine residues in the BSA molecule (*14*), in which 26 residues exist on the surface (*5*). The results show that the number of CPFX molecules did not increase linearly with the increase of the reaction ratio between CPFX and BSA; however, the number of CPFX bound to each BSA molecule can reach as high as 30 (Table 1). It can be explained that the BSA molecule exists in an isomeric structure with high helicity in the environment at pH 5.0 (*15*). This structure contains a part of lysine, which normally exists in the inner part of the protein, exposed on the surface of the carrier protein molecule. Another explanation could be that the hapten can couple with other amino residues besides lysine.

The maximum absorption wavelength in the spectrogram can be used as the basis for the characteristic group analysis of the conjugates. Compared to the characteristic absorption peaks of the carrier protein and hapten, the ones of the conjugates (C1-C5) were shifted (data not shown). The results, which indicate the presence of distinctive groups in the

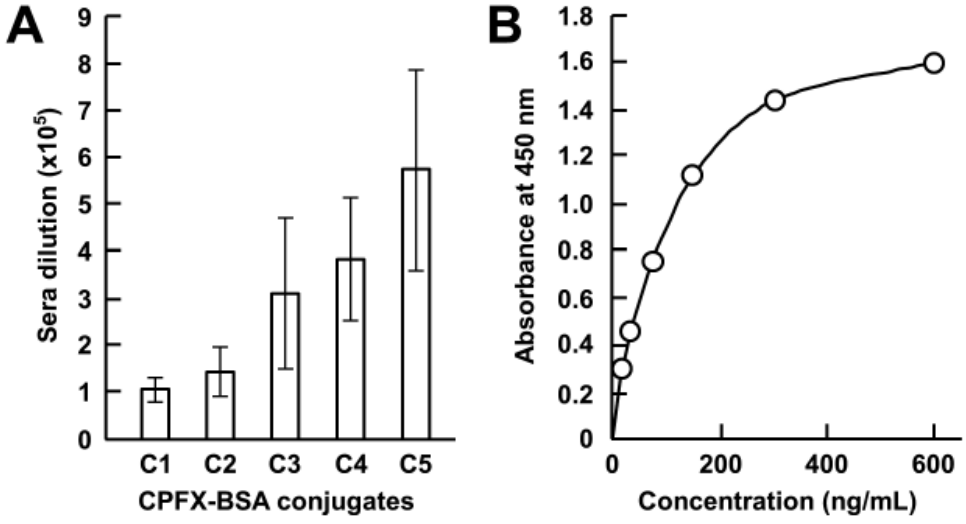

Figure 1. Qualitative analysis of the antibodies generated by various CPFX-BSA conjugates. (A) Titers of antiserum against CPFX obtained from mice immunized with CPFX-BSA conjugates with various hapten densities. Mice were immunized with C1, C2, C3, C4, and C5 having average hapten densities of 21, 25, 26, 28, and 30, respectively. **(B)** Standard dilution curve analysis of antibody generated by the conjugate C5.

conjugates, could confirm the specific binding of BSA and CPFX. The UV spectra of the conjugates suggested that the increase of the hapten-protein ratio caused the absorbance of the conjugate to increase gradually. This also validated the conclusion that the hapten density increased with the increase of hapten-protein ratio.

The amount of hapten bound to the carrier protein is an important factor which affects the quantity and quality of the antibody produced by the conjugate. However, the titer and affinity of the antibody did not increase linearly with the increase of the amount of the hapten bound to carrier protein (*1*). Generally, the binding affinity was optimal when 15 to 30 hapten molecules had bound to the surface of the carrier protein (*5*). The greater amount of hapten bound to the carrier protein, the higher, stronger and more specific antibody titer was produced by their conjugates. However, the immune reaction induced by the conjugate bound with less hapten was slow while the antibody produced by it had a higher affinity. The acquirement of optimal hapten density was important for the conjugate in the preparation of antibody. In addition, the antisera displayed better sensitivity than previously published (*2*). These findings could contribute to improving immunoassay methods.

In conclusion, the protein-hapten mole ratio could increase the hapten density of conjugates, which has a great influence on the immunogenicity of CPFX. An optimum molar ratio of CPFX-BSA conjugates could induce antibodies with good sensitivity, which was suitable to be applied for immunization purposes.

Acknowledgements

This study was supported by the 863 Program (Grant No. 2011AA10A216) and Special Fund for Agro-scientific Research in the Public Interest (Grant No. 201203085).

References

1. Erlanger BF. The preparation of antigenic hapten-carrier conjugates: A survey. Methods Enzymol. 1980; 70:85-104.
2. Duan JH, Yuan ZH. Development of an indirect competitive ELISA for ciprofloxacin residues in food animal edible tissues. J Agric Food Chem. 2001; 49:1087-1089.
3. Fuentes M, Palomo JM, Mateo C, Venteo A, Sanz A, Fernández-Lafuente R, Guisan JM. Optimization of the modification of carrier proteins with aminated haptens. J Immunol Methods. 2005; 307:144-149.
4. Marco MP, Gee S, Hammock BD. Immunochemical techniques for environmental analysis II. Antibody production and immunoassay development. Trends Analyt Chem. 1995; 14:415-425.
5. Singh KV, Kaur J, Varshney GC, Raje M, Suri CR. Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. Bioconjug Chem. 2004; 15:168-173.
6. Hernandez M, Aguilar C, Borrull F, Calull M. Determination of ciprofloxacin, enrofloxacin and flumequine in pig plasma samples by capillary isotachophoresis-capillary zone electrophoresis. J Chromatogr B Analyt Technol Biomed Life Sci. 2002; 772:163-172.
7. Diario Oficial de las Comunidades Europeas (DOCE), August 18, 1990. Council Regulation No. 2377/90 L224, 991, 2601.
8. Zhou Y, Li Y, Wang Z, Tan J, Liu Z. Synthesis and identification of the antigens for ciprofloxacin. Chinese Journal of Veterinary Science. 2006; 26:200-203. (in Chinese)
9. Jiang Y, Huang X, Hu K, Yu W, Yang X, Lv L. Production and characterization of monoclonal antibodies against small hapten-ciprofloxacin. Afr J Biotechnol. 2011; 10:14342-14347.
10. Hua K, Huang X, Jiang Y, Fang W, Yang X. Monoclonal antibody based enzyme-linked immunosorbent assay for the specific detection of ciprofloxacin and enrofloxacin residues in fishery products. Aquaculture. 2010; 310:8-12.
11. Huang X, Hu K, Fang W, Jin Y, Yang X. Preparation of ciprofloxacin-carrier protein conjugation and identification of its products. Journal of Shanghai Fishery University. 2008; 17:585-590. (in Chinese)
12. Habeeb AF. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal Biochem. 1966; 14:328-336.
13. Pingbo Z, Yamamoto K, Wang Y, Banno Y, Fujii H, Miake F, Kashige N, Aso Y. Utility of dry gel from two-dimensional electrophoresis for peptide mass fingerprinting analysis of silkworm protein. Biosci Biotechnol Biochem. 2004; 68:2148-2154.
14. Hirayma K, Akashi S, Furuya M, Fukuhara K. Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and Frit-FAB LC/MS. Biochem Biophys Res Commun. 1990; 173:639-646.
15. Rosenoer VM, Oratz M, Rothschild MA, eds. Albumin Structure, Function and Uses. Pergamon, New York, USA, 1977.

(*Received September 19, 2011; Revised November 29, 2011; Re-revised February 22, 2012; Accepted March 9, 2012*)