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Development of a Method for Pb Functionalization and Preparing Immunogenic Complex

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Abstract

Antibody based methods have numerous advantages compared to other detection methods. However, heavy metals cannot stimulate the immune antibody response; it is the main obstacle for preparation of the antibodies that used in detection methods of the metals. The production of the immunogenic Pb complexes by using cost benefit linkers of EDTA and DTPA were investigated in this study.BSA molecules were functionalized using EDTA and DTPA linkers in various BSA/linker ratio, pH, incubation times and buffers. The complexes were formed after addition of the Pb to the [BSA-linker]. Different concentrations of the glutaraldehyde were mixed with the formed complexes and shaken at room temperature for 12 hours. The prepared conjugates were dialyzed in phosphate buffer saline for 72 hours. Stimulation of the mice antibody responses against the prepared [Pb-linker-BSA] complex were evaluated using ELISA. In optimized condition, the DTPA linker coupled Pb to each BSA molecules 2 times higher than EDTA. The optimum pH was 9.6 in EDTA binding to BSA; also, EDTA molarity was 25 times higher than Pb molarity in [Pb-EDTA-BSA] complexes. These conditions for DTPA conjugation to Pb and BSA are pH 7.4-9.6 and 9.6 respectively. Also, optimum molarity of DTPA was 4 times higher than Pb in [Pb-DTPA-BSA] complexes. Also, the higher antibody responses against Pb were stimulated by immunization

with an antigenic complex that has more solubility and Pb coupled to each albumin molecules. In conclusion, in optimized conditions, DTPA more efficient than EDTA for synthesizes of the immunogenic Pb complex.

Keywords: Antibody, DTPA, EDTA, Pb, Linker.

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Introduction

Heavy metals are considered as agents of harmful toxicity. Food and water contaminated anywhere that has high levels of heavy metals. Heavy metals accumulation may cause severe or chronic toxicity [1, 2]. Humans often consumed the heavy metals through contaminated plants and animal products [3]. Higher occurrence of toxicity occurred through using pots, dishes and fossil fuels which contain heavy metals. These metals are not metabolized, but they remained and accumulated in the body; their attachments to the cell receptors initiated the toxic effects [4]. Heavy metals may interfere with nucleus proteins and DNA; it causes oxidative degeneration of macromolecules [5].

Lead is a toxic heavy metal that can be found everywhere; the symptoms of lead poisoning had been discovered first by Hippocrates several centuries ago [6]. Lead poisoning is one of the oldest environmental and occupational diseases in the world [7].

Contact with the lead is inevitable; it is existed in organic and non-organic materials; human often inhale air which containing Pb [8]. In addition, lead toxicity has numerous toxic effects especially on immunity, hematopoietic, central nervous system, liver and kidney [9]. Although lead poisoning has been broadly studied, the control and prevention of its poisoning may be impossible especially in developing countries.

The general detection methods of heavy metals are atomic absorption spectroscopy, mass spectroscopy, ICP-MS, induction plasma mated, ICP-OES and x-ray fluorescence. These methods are often costly, time-consuming and require highly specialized devices and professional staffs; so they are not appropriate methods for the rapid, easy and inexpensive detection of the heavy metals [10].

Antibody-based approach offers a useful method for lead detection. This method has significant advantages compared with usual techniques; it is quick, inexpensive, simple, portable, highly sensitive and selective experiment. Antibody based methods could decrease analysis cost 50% or more [11]. Because of the low molecular weight, heavy metals have a simple structure which categorized as hapten and usually cannot stimulate the immune responses. Therefore, the key step in heavy metals detection using antibody based methods is production of the immunogenic antigen [12].

The immune responses are more stimulated against molecules which have high molecular weights, complex structure and a high degree of alienation. Heavy metals with low molecular weight could not stimulate the immune responses; however, it could be generated by heavy metals that coupled to an immunogenic protein. Initially, the heavy metals must couple to a carrier protein through linker molecules as ethylene diaminetetraacetic acid (EDTA) and diethylene triamine penta acetic acid

(DTPA). This research aimed to evaluate conjugation of the lead to bovine serum albumin in order to generate antibody immune responses in mice.

Experimental

Materials

This research was conducted by using Lead (II) acetate (Pb) (Merck, 32306), Diethylene triamine pentaacetic acid (DTPA) (Sigma, D6518), Ethylene diamine tetraacetic acid (EDTA) (Merck, E6758), Glutaraldehyde (Merck, G6257), Bovine serum albumin (BSA) (Sigma, 05470), conjugated anti-mouse antibody (Sigma, A5278), Freund´s adjuvant (Razi vaccine and serum research institute, Iran), *N*,*N*′-Methylene bis acrylamide (Sigma, M7279), Acrylamide (Sigma, A2917), Ammonium per sulfate (Sigma, A3678), *N*,*N*,*N*′,*N*′-Tetra methyl ethylene diamine (TEMED) (Sigma, T9281), Tris base (Merck, 648310-M), Sodium dodecyl sulfate (SDS) (Sigma, L3771), Glycine (Sigma, 1.04169) and Coomassie brilliant blue R250 (Sigma, 1.12553).

[Pb-EDTA-BSA] and [Pb-DTPA-BSA] preparation

The [Pb-EDTA-BSA] and [Pb-DTPA-BSA] complexes were synthesized based optimization of pH, Pb/linker and Pb/BSA ratio, buffers and dialysis; also, and the effects of glutaraldehyde levels on the complexes formation were evaluated [13, 14, 15]. In brief, BSA molecules were functionalized using EDTA and DTPA linkers in various BSA/linker ratio, pH, incubation times and buffers. The prepared [BSA-linkers] were dialyzed in buffers for 48 hours. The complexes were formed after addition of the different levels of Pb to the [BSA-linker].

Different concentrations of the glutaraldehyde were mixed with the formed complexes and shaken at room temperature for 12 hours. The prepared conjugates were dialyzed in phosphate buffer saline for 72 hours. The control samples including [BSA-EDTA] and [Pb-EDTA] were prepared as the optimized conditions. The protein content of the prepared complexes was assessed using Bradford method.

The patterns of the BSA bands in the prepared complexes were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels prepared as 12% separating gel and 4% stacking gel, electrophoresed in running buffer (25 mM Tris base pH 6.8, 192 mM glycine, 1% SDS) at 100V for 90 minutes. The polyacrylamide gels were stained for 30 minutes with Coomassie staining solution followed by de-staining with 7% acetic acid solution overnight. The produced complexes were stored at 4°C. The conjugates stability was tested during two months using SDS-PAGE.

Atomic absorption analysis

Based on the SDS-PAGE results, six samples were selected among all of the produced hapten-carrier complexes. The accurate removal of the free Pb was achieved by additional dialysis against phosphate buffer for 72 hours at 4°C, with buffer change every 24 hours. The lead content of the prepared six samples was measured by using atomic absorption spectrophotometer (Shimadzu, UV-6200-A) on 283.5 nm. The standard curve (Figure 1) was prepared using lead standard solution (Sigma, NIST3128).

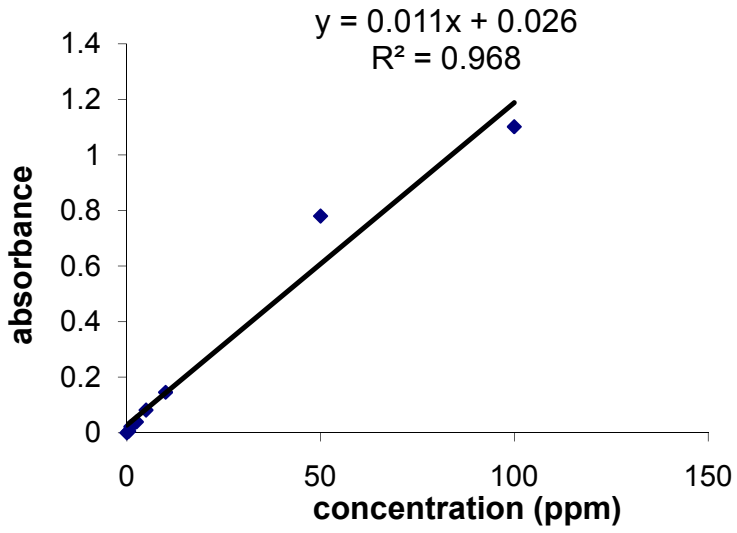

Figure1.The prepared standard curve using atomic absorption spectrophotometer for detection of the lead levels on [pb-linker-BSA] complexes.

Immunization

Based on the atomic absorption results, three samples that had the highest Pb/BSA ratio were selected for immunization step of the experiment. The antigenic complexes were injected subcutaneously to three groups including 6 male, albino mice with the mean weight of 24±2 gr. The ethics of using animals in experiments were approved by Animals Ethics Committee (AEC) of the Veterinary Medicine Faculty of Shahid Chamran University of Ahvaz, Iran. The immunization was conducted by injection of the conjugates (50 µl) mixed with complete Freund´s adjutant (50 µl); Three sets of booster which contained the same amount of the conjugates and incomplete Freund´s adjutant were injected with 2 weeks intervals. The control group received unconjugated Pb+BSA with the same route of antigen preparation and injections. The hyper-immune sera were collected by cutting the end part of the mice tail.

Results and Discussion

Pb coupling to BSA

This experiment optimized the stable coupling of Pb to BSA using DTPA and EDTA linkers. The Pb/linker ratio was evaluated on 2.1, 4.1 and 8.1 and greater. The reactions were performed on neutral and alkaline pH conditions. The PEGylation and dialysis was equipped for the proper solubility and stability of the coupling. The results showed that Pb salts which dissolved in phosphate buffer (pH: 8.3), conjugated to EDTA on pH: 9.6. The optimum amount of EDTA was 25 times greater than the used Pb molarity. Bovine serum albumin had optimal attachment to the [Pb-EDTA] on pH: 9.6. Also, the BSA and DTPA had the finer reaction on pH: 9.6. The [Pb-EDTA-BSA] and [Pb-DTPA-BSA] complexes were prepared by adjustment the BSA/Pb proportion on 1/10 and 1/100, respectively.

The Pb molecules reacted with [DTPA-BSA] on pH ranged from 7.2 to 9.6. The required amount of DTPA was 4 times greater than Pb. In addition, the tris-HCl buffer pH 9.6 was an appropriate buffer for BSA coupling to DTPA. The appropriate pH for Pb coupling to DTPA-BSA complex was 8.2. This is in agreement with Xiang et al. [12] study.

Different pH was reported for EDTA binding to Cd (pH: 7.5), Cr (pH: 7), Mercury (pH: 7.0) and Pb (pH: 7.5) [16-19]. In Zhu et al. [20] study the glutaraldehyde concentration was equal to 2.5 mm; however, in the current experiment the optimum amount was equal to 8.3 mM. The glutaraldehyde amount higher than 8.3 mM, would reduce the [Pb-DTPA-BSA] complex formation. However, different materials and methods may affect on the required pH for reactions aptness.

Previously, DTPA linker was stably linked to several metals such as Zn^{2+}, Mg^{2+}, $Mn2^{+}$, Co^{2+}, Cu^{2+}, Pb^{2+}, Ca^{2+} and the radio-nucleotides; the ring structure of the DTPA is a suitable region for binding to heavy metals [21]. In some efforts, the stable attachment of Pb to carrier's protein was not achieved using EDTA linker [22]. However due to the economical issues and the availability, EDTA is considered as one of favorable linker [16].

The obtained SDS-PAGE showed development of the high molecular weight bands on optimal conditions. The other tested conditions for Pb conjugation had little or no effects on the pattern of these bands. Also, excellent stability was observed at least for two months (Figure 2).

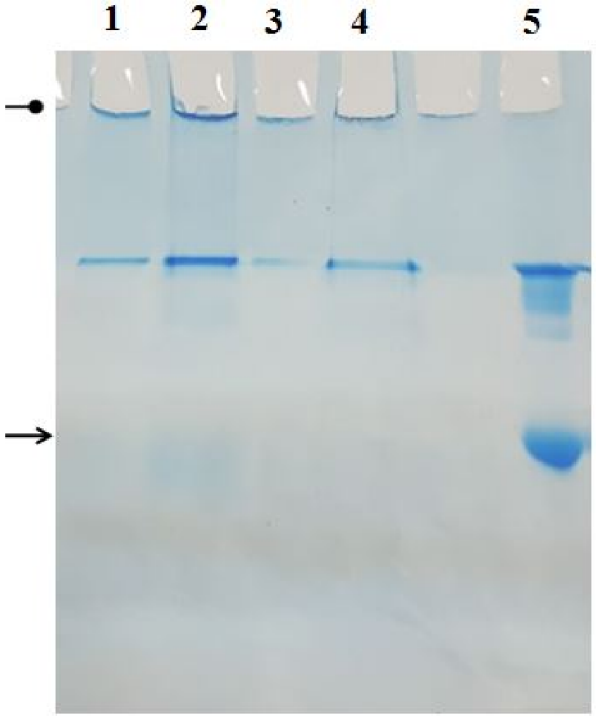

Figure2. Analysis of the [Pb-EDTA-BSA] and [Pb-DTPA-BSA] stability using Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). 1 and 2) the prepared conjugates using EDTA linker and 3 and 4) the prepared conjugates using DTPA linker according to the optimum conditions; 5) BSA. Arrow shows the free BSA ( ) band and the conjugated BSA in high molecular weight complexes which could not penetrate to the gel ( ). Existence of high molecular weight bands and decreasing BSA bands were observed until two months after production of the conjugates.

The prepared conjugates initially were evaluated using SDS-PAGE. Six samples were selected to detect Pb content using the atomic absorption. Based on the atomic absorption results (table 1), the equal quantity of lead was coupled to BSA on samples 1, 2 and 3.

Table 1. The atomic absorption test for detection of the Pb levels in the prepared [Pb-linker-BSA] complexes. The 1, 2, 4 and 5th samples were prepared using DTPA linker; the 3 and 6th samples was equipped using EDTA linker.

Samples	(µg/ml) Pb level	BSA/Pb(%)
1	329.41	12.7
2	403.34	12.3
3	395.995	23
4	217.935	22.9
5	6.65	751.8
6	48.4	127

Table 2 shows the interpretation of the ELISA results. These results indicated production of the antibody against Pb, [Pb-EDTA-BSA] and [Pb-DTPA-BSA] complexes. The highest Pb antibody titer was produced in groups 1, 3 and 2, respectively.

Table 2. The ELISA result (mean optical density ± standard deviation) of the antibody immune responses against conjugates and Pb. The wells coated with the antigen complexes that had been used for immunization. The control wells were prepared by mixing each sample with Pb: the results noted on (Pb-linker-BSA)-Pb column; or BSA-linker: the results noted on BSA-linker column.

Conjugate	Pb-linker-BSA	BSA-linker	(Pb-linker-BSA)-Pb
1	0.430±0.141	0.097±0.039	0.256±0.136
2	0.163±0.030	0.058±0.018	0.103±0.047
3	0.207±0.05	0.052±0.004	0.135±0.013

Table 3 shows the interpretation of the obtained results. Elevation of optical density of the test wells compared with the control wells showed the production of antibody against Pb, Pb-EDTA-BSA and Pb-DTPA-BSA conjugates.

Table 3. The ELISA result (mean optical density ± standard deviation) of the antibody immune responses against Pb. The microplate wells were coated with [Pb-DTPA-BSA]; the control wells were prepared by encountering each sample with Pb: the results noted on Pb-linker-BSA)-Pb column; or BSA-linker: the results noted on BSA-linker column. All samples were mixed with the same amount of BSA-DTPA.

Conjugate	Pb-DTPA-BSA	BSA-linker	(Pb-linker-BSA)-Pb
1	0.286±0.164	0.148±0.108	0.189±0.103
2	0.382±0.110	0.190±0.027	0.286±0.111
3	0.291±0.105	0.187±0.74	0.234±0.100

The findings indicated that DTPA linker have more ability to couple Pb to BSA molecules than EDTA. Stronger induction of the humoral immune responses may be related to the greater amount of Pb that existed in [Pb-DTPA-BSA] complex than the prepared conjugates on the other conditions. The immunogenicity of the coupled antigen is dependent to the hapten density on the carrier protein. The preparation conditions of the various conjugations have different effects on conformational change of the carrier protein; previously, diverse carrier/hapten ratios were reported [23].

The high hapten conjugation ratio may induce the more low affinity IgM response. In current study, EDTA, DTPA and glutaraldehyde were utilized as short cross-linker reagents to aid Pb attachment to BSA molecules. This method produced a stable cross-linking of Pb to BSA along with acid

amide and carbons bridge formation with satisfactory IgG responses subsequent to immunization. The other linker included 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N, N-dicyclohexyl-carbodiimide (DCC), N-hydroxysuccinimide (NHS) routinely used for preparation of the heavy metals immunogens [24, 25]; the current study optimized production of the stable complex of antigen by using more cheaply linkers. Previously, researcher used DTPA for extraction of the heavy metals from soil [26] and plants [27].

The free linkers and Pb were removed from the reactions through two steps of dialysis 1-after attachment of Pb or BSA to linker 2- after preparation of [Pb-linker-BSA].

The appropriate levels of linker were selected based on the observed changes on the SDS-PAGE pattern of BSA bands. In optimum points, no further changes were observed on the bands pattern by increasing amounts of linker or Pb. The conjugates without dialysis, which prepared according to optimum conditions, contain appropriate amounts of Pb; but it has less solubility than the dialyzed samples. Immunization of the mice with prepared conjugate without dialysis resulted in less antibody titer production than the other conjugates. It concluded that the conjugates solubility is an important aspect regarding with the immunogenicity.

Conclusion

In conclusion, in optimized conditions, DTPA more efficient than EDTA for synthesizes of the immunogenic Pb complex. According to the results, three conditions resulted to the appropriate conjugation. The resulted complexes have different properties based on the level of the coupled Pb, BSA and solubility; these characters have main effects on the Pb complex immunogenicity.

Conflict of interest

Author declares that have no conflict of interest.

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