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RESEARCH ARTICLE

DEVELOPMENT OF FLUORESCENCE IMMUNOASSAY BASED ON CADMIUM SULPHIDE (CDS) QUANTUM DOTS FOR THE DETECTION OF ENDOSULFAN

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ABSTRACT

Introduction: Cadmium sulfide quantum dots were used for a sensitive detection of endosulfan. Cadmium sulfide (CdS) quantum dots (QDs) (nanoparticles) were synthesized, purified and attached to anti-endosulfan IgY antibodies for the detection of endosulfan. Followed by the concentration of antibody optimization, the fluorescence-linked immunoassay method was based.

Results: The method used the QDs as the signal to quantify endosulfan. Compared with the chromogen based enzyme-linked immunosorbent assay (ELISA), QD-based ELISA saved time and decreased the false-positive results because of specified emission wavelength of QDs. The 50% inhibitory concentration (IC_{50}) of the QD-ELISA method was 0.28 mg mL^{-1} with a detection limit of 25 ng mL^{-1},and the linear range was 60ngmL^{-1}-3.83mgmL^{-1}. Endosulfan residues in spiked samples were also determined. The recovery of endosulfan in water samples ranged from 84.5% to 96.2% and that in vegetables ranged from 72.5% to 125.7 %.

Conclusion: Among food matrices used, Lemon Rice interfered in the quantitative detection of endosulfan. However, the detection of the endosulfan residues in the environment and food samples is rapid, sensitive and accurate.

INTRODUCTION

Increased use of pesticides and fertilizers [1] has led to increased agricultural productivity that has resulted from the Green Revolution. It has caused high yields and increased crop productivity, although the overuse of pesticides and herbicides has caused potentially harmful environmental effects. Pesticides have been used globally on a large scale in agriculture, food storage facilities, and even in public health operations aimed at controlling weeds, insects, and rodents [2].

Among pesticides, organochlorine pesticides are used extensively to prevent crop loss due to stem borer, grey weevil, brown plant hoppers, and many other pests that would otherwise cause enormous crop losses. The widespread use of these pesticides has resulted in significant groundwater contamination. It is due to their accumulation in the food chain [3], which has filtered through to various food formulations and drinking water [4],[5],[6].

Endosulfan**,** an organochlorine insecticide and an acaricide introduced in the 1950s is being slowly phased out globally of which the two isomers, endo, and exo are known popularly as Alpha(α) and Beta(β). Endosulfan became a highly divisive agrichemical [7] due to its acute toxicity, potential for bioaccumulation, and role as an endocrine disruptor. This organochlorine insecticide is a highly lethal, ubiquitous environmental pollutant, causing long-term effects to human and wildlife. It is typically considered to be a Persistent Organic Pollutant (POP), included in the initially proposed list of POPs. Because of its threats to human health and the environment, a global ban on the manufacture and use of endosulfan was negotiated under the Stockholm Convention in April 2011. Although classified as a yellow label (highly toxic) pesticide by the Central Insecticides Board, India is one of the largest producers [8] and the largest consumer of endosulfan in the world [9].

The Major Endosulfan (Technical grade) manufacturers in India are Excel Crop Care, Hindustan Insecticides Ltd, and Coromandel Fertilizers.They produced 9,500 MT (Million Tons), of which 4,000 MT was for exporting in 2007-08 [8]. Despite imposing restrictions on it, since 1996-97, India produces an average of 8206 MTPA (Million Tons Per Annum) totaling 41033 MT between 1995 -2000. India has exported 12180 MT during this period and consumed on an average 3599 MTPA [10].The widely used Endosulfan is in most of the plantation crops in India.

The half-life of endosulfan in soil varies from 60 days (α-endosulfan) to 800 days (β- endosulfan), with persistence

increased by acidic conditions. The half-life in water ranges from 35-150 days [11]. Residues have been detected in air, water bodies, river sediment, soil, tree bark, aquatic plants and various biota [12].They find their presence in dairy foods, meat, chicken, vegetable oil, peanuts, seeds and other food samples. Thus, it enters the human body *via the* food chain. It is found to be positive in human tissues, in blood samples, human sera, adipose tissue, human milk, urine samples [10]. It is highly poisonous to cats (LD_{50}-2mg/kg) and dogs (LD_{50}-76.7mg/kg) [13]. Due to its long environmental fate, it is necessary that proper detection methods be available for faster, specific, sensitive and easier detection of endosulfan.

Developments in smart nanomaterials, such as quantum dots (QDs) and gold nanoparticles (GNPs), have gained much interest from biological researchers because of their unique spectral properties [14]. QDs exhibit exclusive optical and electronic properties based on the firm confinement of excited electrons and their corresponding holes (called excitons) within their structures [13].QD emission is size dependent, and so the emission properties of the QDs can be altered by engineering their size.

The unique optical properties of these aqueous synthesizedQDs, such as photostability for extended periods of time, broad absorption, narrow and very specific, stable emission spectra. This property along with biocompatibility [15],[16], have led to them becoming of great interest for biological labeling [17], [18].

Hence, the objective of this paper is to investigate the potential immunoassays based on QDs for the detection of endosulfan residues in spiked food and other environmental samples. The QD ELISA method involves CdS QDs as the fluorescent marker is described and applied to different food matrices.

MATERIALS

Cadmium chloride ($CdCl_2$. $2.5H_2O$ > 98%), sodium sulphide (Na_2S. $9H_2O$, 98.0%) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxy Succinimide (NHS) were purchased from Sigma-Aldrich Co. (USA). Ethylenediamine and 3-mercaptopropionic acid ($HSCH_2CH_2COOH$) were purchased from SISCO Research Laboratories Pvt. Ltd. Mumbai, India. All other chemicals used in this study were of analytical grade and procured from standard chemical companies.

METHODS

Anti-endosulfan antibody

Anti-endosulfan chicken antibody produced followed the method of [19].

Synthesis of Water Soluble CdSNPs

Water soluble CdSNPs were synthesized using the previously published method [20]. Briefly, carboxyl-stabilized CdSNPs were synthesized by arrested precipitation at room temperature in an aqueous solution using mercaptoacetic acid as the colloidal stabilizer. Nanocrystals were prepared from a stirred solution of 5 mM $CdCl_2$ in 40 ml of pure water. The pH was maintained at two by the addition of mercaptoacetic acid and then raised to 7 with concentrated NaOH. N_2 bubbling deaerated the mixture for about 30 min, then, a freshly prepared 40 ml solution of 5 mM Na_2S in water was added to the mixture with rapid stirring. The solution turned yellow shortly after the sulfide addition due to the cadmium sulfide nanoparticles formation (CdSNPs). CdSNPs were separated from reaction byproducts by precipitation with the addition of acetone [21]. Centrifugation isolated the precipitate, which was dried in a freeze dryer.

Conjugation of CdSNPs with anti-endosulfan antibodies

Conjugation of CdSNPs with anti-endosulfan Antibodies was a two-step process. First, with CdSNPs, Ethylenediamine were reacted to introduce amine groups on the surfaces. For that, CdSNPs were dissolved in an aqueous solution containing 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (EDC) and stirred for four h to activate the carboxylic acid groups on the surfaces. Then, the excess amount of Ethylenediamine was added to the solution and stirred for 24 h to obtain primary amine-grafted CdSNPs (ECdSNPs). An excessive amount of ethylenediamine was added in the coupling reaction to keep the amine groups free at one end of ethylenediamine after the reaction. The prepared ECdSNPs were isolated *via* repeated centrifugation and finally dried in a freeze dryer.

In the second step, the antibody was immobilized on the surface of ECdSNPs. Anti-endosulfan antibody was dissolved in a phosphate buffer solution (40 ml, pH 7) containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). This solution was stirred for five hours to activate the carboxylic acid groups of the antibody. Then, ECdSNPs were added to this solution and the mixture was stirred for 48 h at room temperature to obtain antibody-coated ECdSNPs (ACdSNPs). ACdSNPs were isolated by repeated centrifugation and stored in phosphate buffered saline (PBS) at pH 7.8 All the conjugation reactions, unless otherwise noted, were carried out in the dark under an N_2 ambient environment.

Optimization of the concentration of antibody linked with QDs

The concentration of anti-endosulfan IgY antibody was determined via checkerboard method. An antigen at different levels (2 $mgmL^{-1}$, four $mgmL^{-1}$, and eight $mgmL^{-1}$) was coated on the 96-well plate (100 µL per well) at 4ºC overnight. After a threefold washing, gelatin solution (1%) was added to block the wells (100 µL per well) for one h at 37ºC. Moreover, then an antibody at different concentrations (from 1:1 to1:16) was added to the ELISA wells to react with antigen. Then the plates were washed three times with PBST (Phosphate Buffer Saline Tween-20).

The plates were incubated for one hour at 37 ^{0}C. After the last threefold washing, PBST (100 µL) was added to each well. The fluorescence intensity of each well was measured at wavelengths of 510 nm (excitation of 330 nm) at room temperature.

Quantum dot Enzyme-linked immunosorbent assay (QDELISA)

100 µL per well of antigen solution (2 $mgmL^{-1}$) was coated on the plates and incubated overnight at 4ºC. The plates were washed and blocked as mentioned in the above section. After another threefold washing, endosulfan standards of various concentrations (from 100 μgL^{-1} to 1 fgL^{-1} in PBS with 1% (Di-methyl formamide) were added to the wells (100 µL per well) to compete with the antigen to react with the CdS coated antibody solution. Then the CdS coated antibody solution was added (100 µLper well). The concentration of antibody solution was that as optimized in checkerboard analysis. The reaction was carried out for one h at 37ºC. After the last threefold washing, 100 µL of PBS was added to measure the fluorescence intensity (Figure 1).

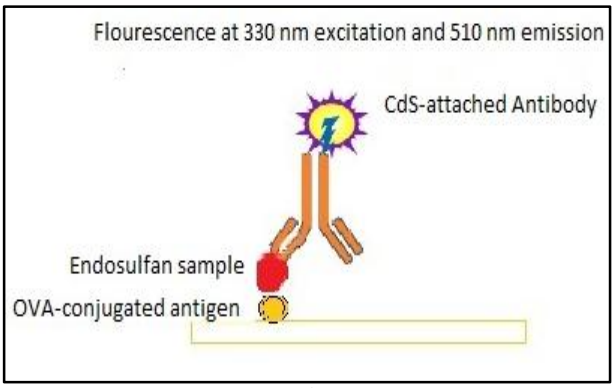

Figure 1. Scheme of QDELISA method

Specificity of antibodies

Few organochlorine pesticides such as 1, 3-dichloropropene, 1, 2, 3-trichloropropane, 1, 4-dichlorobenzene, 1, 2, 4-trichlorobenzene, hexachlorobenzene, methoxychlor, diuron, aldrin, endrin and standards were used for specificity studies.Specificity was calculated as the reduction in activity compared to the control (isomers) and expressed as relative percentage activity. By Dissolving standards, pesticide stock solutions were prepared at one $mgmL^{-1}$ in acetone. Assay standards were prepared by diluting the stock solutions using buffer/dimethylformamide (1: 1) in borosilicate glass tubes and used within 30 min of preparation. Other QDELISA reactions were same as given above.

Food matrix effect in the detection of endosulfan
Analysis of spiked water samples

Water samples collected from different sources were used. They were spiked with 100 ng of α- and β-endosulfan separately and allowed to stabilize overnight. Unspiked water samples were maintained as controls. After stabilization, the spiked and unspiked samples were adjusted to pH 2.0 with concentrated HNO_3 and extracted thrice with three volumes of dichloromethane. The solvent fractions were pooled, passed over anhydrous sodium sulfate in phosphate buffer (50mM, pH 7.5) containing DMF for immunoassay. A QDELISA was performed as described earlier.

Spiking of food samples

Food samples were spiked with known concentrations of α-endosulfan and β-endosulfan, mixed well and kept overnight at room temperature and 4^0C for stabilization. Food samples without spiking served as control. The pH of the spiked and unspiked samples was adjusted to 2.0 with concentrated HNO_3 and extracted thrice with three volumes of dichloromethane. The solvent fractions were pooled, passed over anhydrous sodium sulfate in phosphate buffer (50mM, pH 7.5) containing DMF for immunoassay. The competitive immunoassay was performed out as described earlier.

RESULTS

CdS nanoparticles

The average size of the nanoparticle CdSNP has been found to be 2.525 nm by X-ray diffraction studies**.**The fluorescence spectra (excitation at 401 nm) of CdSNPs and ACdSNPs indicated that the fluorescence intensity of ACdSNPs increased significantly compared to CdSNPs (data not shown) at the same concentration. The fluorescence intensity of ACdSNPs was stronger by ca 1.5 -fold than that for CdSNPs(data not shown), which was demonstrated.

Detection of endosulfan isomers by quantum dot enzyme-linked immunosorbent assay (QDELISA)

The detection of endosulfan isomers by QDELISA was based on an indirect competitive method. Competition between free endosulfan isomer molecules and the quantum dot tagged antibody for the antibody-binding sites in an immobilized antigen was the key reaction for the sensitive detection of endosulfan isomers in water and food samples. Hence, for less free endosulfan isomer concentration in the sample, more anti body would bind to the antigen (Fig.1).

Thus, the fluorescence intensity obtained are inversely proportional to the concentration of free endosulfan isomers in the sample. The results were compared with a negative control that gave maximum fluorescence signals. Glutathione-coated CdS nanoparticles were used in the detection of dicofol and dimethoate (22). However, this method was used for direct detection without conjugating them to antibodies.

The optimization of antigen and anti body concentrations.

The optimum concentrations of antigen and antibody required was done by Checkerboard analysis which determined the best working concentration of the conjugate before coating onto the microwell plates. The analysis indicated that antigen (Alkaline phospatase) conjugate dilution of 1:25000 with carbonate buffer and antibody dilution of 1:10000 gave the optimum readings. (Data not shown) These concentrations were chosen for further work.

THE DEVELOPMENT OF QDELISA METHOD

α- concentrations from 10µg to 1fg and β- concentrations from 1µg to 1fg were tested to obtain the minimum and maximum detectable levels. Competitive ELISA was performed, and the residual fluorescence intensity was detected.

Figure 2 & 3 showed the effect of endosulfan on QDELISA with IgY. Up to 1fg was analyzed by this method. There was 97% inhibition at 10µg of the α-isomer. Percent inhibition

was 13% at 1fg of the α-endosulfan. β-endosulfan induced 97% inhibition at 1µg concentration and at 1fg level the inhibition was 8%.

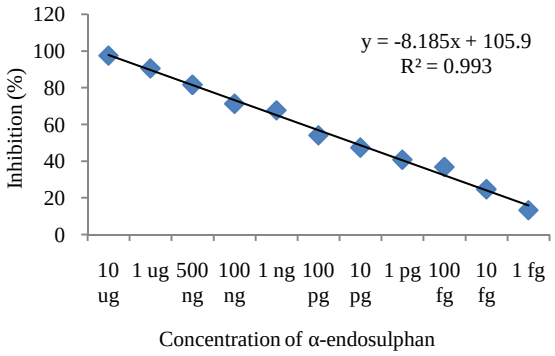

Figure 2 α-Endosulfan detection levels using CdS Nanoparticles

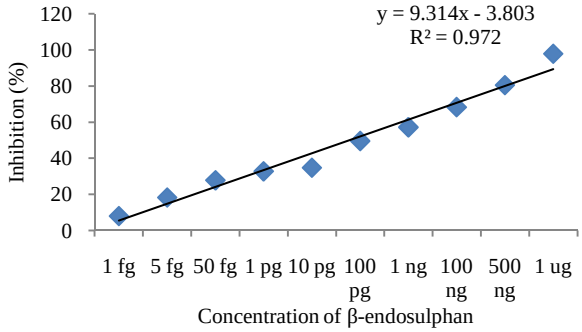

Figure 3 β-Endosulfan detection levels using CdS Nanoparticles

DISCUSSION

Cross-reactivity

Eleven different organochloride pesticides were used to measure the cross-reactivity (CR) between the QD-tagged antibody and other OCPs to study the specificity of the method. It was observed that the anti-endosulfan antibody was specific, and no other OCPs reacted with antibody (Table 1)

Table 1 Cross-Reactivity of QD-antibody with other OCP s.

Sl.No.	Pesticide used	IC_{50} value
1	α- endosulfan	92.57pg
2	β- endosulfan	101.07 pg
3	1,3-dichloropropene	166.24 µg
4	1,2,3-trichloropropane	350.41 µg
5	Dichlorobenzene	264.27 µg
6	Trichlorobenzene	74.54 µg
7	Hexachlorobenzene	66.7 µg
8	Methoxychlor	228.4 µg
9	Diuron	0
10	Aldrin	0
11	Endrin	295.66 µg

Note: Few pesticides were tested for cross-reactivity to the anti-endosulfan antibody. Data were based upon the mean of triplicate determinations. Competitive immunoassays were performed as described in the experimental section using analytical grade standard pesticides.

Precision and accuracy of the assay

The inter- and intra-assay precisions were determined at different endosulfan isomer concentrations, 10µg to 1fg mL^-1for α-endosulphan and 1µg to 1fg for β-endosulphan. The intra-assay precision was assessed by analyzing eight replicates of each sample in a single run, and interassay precision has been evaluated by analyzing the same by repeated analysis of samples of endosulfan isomers (Table 2). The coefficient of variation of five assays of each standard was satisfactory, with less precision at lower analyte (endosulfan isomer) concentrations.

Table 2 Precision of QDELISA assays

Concentration / mL	SD α-endosulphan		SD β-endosulphan	
	Inter-assay	Intra-assay	Inter-assay	Intra-assay
1fg	0.087	0.091	0.081	0.077
10fg	0.035	0.046	0.041	0.035
100fg	0.045	0.044	0.032	0.042
1pg	0.036	0.023	0.034	0.021
10pg	0.028	0.032	0.056	0.045
100pg	0.036	0.042	0.035	0.042
1ng	0.033	0.026	0.032	0.021
10ng	0.048	0.044	0.029	0.028
100ng	0.043	0.056	0.064	0.052
1µg	0.057	0.042	0.061	0.054
10µg	0.054	0.048		

SD - standard deviation

Analysis of spiked samples

Different food matrices such as vegetables, cooked foods, fruits, bread were checked for the interference of the matrix in CdS quantum dot based immunoassay for the detection of endosulfan. The studies were conducted using both α-endosulfan and β-endosulfan (Table 3). Most of the vegetables showed good recovery of α-endosulfan. Recovery was slightly lower with β-endosulfancompared to that with the α-isomer. Among vegetables, garlic matrix showed 99% recovery of α-endosulfan followed by beetroot. Carrot showed the lowest recovery of 74 % of α-endosulfan and ginger showed 104 % Recovery. Lemon rice showed 114% recovery indicating matrix interference. For β-endosulfan analyte, lemon showed 95% recovery followed by a carrot. Apple showed 102 % recovery. The overall recovery ranged from 74 to 114 % for α- endosulfan and 70% to 102% for β-endosulfan.

Table 3 Recovery Percentage of Endosulfan from Spiked foods

Sl. no.	Food sample spiked	Recovery of α- endosulfan (%)	Recovery of β- endosulfan(%)
1.	Onion	85.29	74.46
2.	carrot	74.01	86.3
3.	Butter	83.69	79.22
4.	Lemon	78.61	95.67
5.	Chilli	81.87	75.78
6.	Bread	88.99	82.91
7.	Ginger	104.6	80.93
8.	Garlic	99.77	73.46
9.	Tomato	83.82	75.36
10.	Ladies finger	83.65	70.37
11.	Beetroot	91.31	71.62
12.	Cabbage	85.68	73.02
13.	Lemon rice	114.73	77.24
14.	Apple	81.24	102.38

CONCLUSIONS

The CdS quantum dot based immuno-technique has been employed for the sensitive detection of isomers.Linearity was in the range of 10 μgmL^{-1} –1 $fgmL^{-1}$ with α-isomer and 1μgmL^{-1} –1 $fgmL^{-1}$ with β-isomer. The results obtained indicated that CdS quantum dot based technique could be effective as a quantitative test for the detection of isomers. This method is suitable for rapid and reliable detection of

isomers in water and food samples. The method provides preliminary, quantitative data, which can be applied to the analysis of environmental samples. These techniques may help in the biomonitoring of isomers in a given sample without significant matrix interference

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