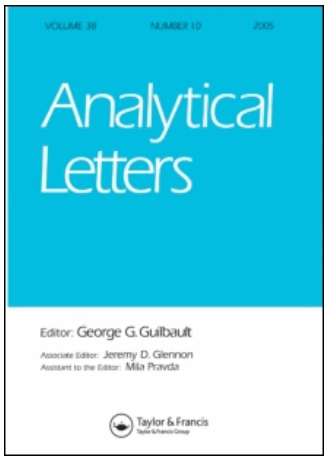

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IMMUNOLOGY

Quantum Dots as Reporters in Multiplexed Immunoassays for Biomarkers of Exposure to Agrochemicals

M. Nichkova, D. Dosev, A. E. Davies, S. J. Gee, I. M. Kennedy, and B. D. Hammock
University of California, Davis, California

Abstract: The application of quantum dots (QDs) as labels in immunoassay microarrays for the multiplex detection of 3-phenoxybenzoic acid (PBA) and atrazine-mercapturate (AM) has been demonstrated. PBA and AM are biomarkers of exposure to the pyrethroid insecticides and to the herbicide atrazine, respectively. Microarrays were fabricated by microcontact printing of the coating antigens in line patterns onto glass substrates. Competitive immunoassays were successfully performed using QDs (QD560 and QD620) as reporters. The multiplexed immunoassays were characterized by fluorescence microscopy and SEM. The application of QD fluorophores facilitates multiplex assays and therefore can contribute to enhanced throughput in biomonitoring.

Keywords: Quantum dot, microarrays, immunoassay, microconatct printing, pyrethroid, atrazine

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Address correspondence to B. D. Hammock, Department of Entomology, University of California, Davis, 95616 California. E-mail: bdhammock@ucdavis.edu

INTRODUCTION

The microarray format has become an important tool for parallel (or multiplexed) monitoring of biomolecular interactions. Protein microarray technology plays a fundamental role in the miniaturization of biosensors, proteins analysis, drug screening, clinical immunological assays, and biomarker identification (Kusnezow and Hoheisel 2002; Espina et al. 2003). Although microarrays are usually visualized and analyzed using organic fluorescent dyes (Wiese 2003; Espina et al. 2004), their poor photostability, the decreasing of the quantum yields upon conjugation to biomolecules and the self-quenching at high labeling ratios limit their effectiveness in such applications. New fluorescent probes for microarray-based bioanalysis such as europium chelates (Scorilas et al. 2000), fluorophore loaded latex beads (Orth, Clark, and Craighead 2003), dye-doped silica nanoparticles (Zhou and Zhou 2004; Yao et al. 2006), lanthanide oxide nanoparticles (Dosev et al. 2005; Nichkova et al. 2006) and inorganic nanocrystals (quantum dots, QDs) (Gerion et al. 2003; Geho et al. 2005) have been explored. QDs are very attractive for biolabeling due to their properties such as excellent brightness, narrow and precise tunable emission, negligible photobleaching, fairly high quantum yields, and photostability (Bruchez et al. 1998; Chan and Nie 1998). The potential of QDs to address "multiplexing" has already been demonstrated for biological imaging (Chan et al. 2002) and toxin analysis in a single well of a microtiter plate (Goldman et al. 2004). Their broad excitation spectra allow simultaneous excitation of different particle sizes at a single wavelength with emission at multiple wavelengths, each specific for an individual QD fluorophore. QD fluorophores enable multiplex assays requiring a single excitation source far from the QD emission peaks. Although QDs have proven to be suitable labels in bioanalysis, their application in quantitative immunoassays for small molecules is still very limited (Goldman et al. 2002; Ding et al. 2006).

Microcontact printing (μCP) is a relatively new method for chemically and molecularly patterning surfaces on a submicrometer scale (Kane et al. 1999). It has been successfully applied to the direct patterning of proteins on a micrometer scale without loss of their biological activity on a variety of surfaces (Bernard et al. 2000; Tan, Tien, nd Chen 2002; Graber et al. 2003). The low cost of fabrication and the simplicity of transferring proteins to substrates, when compared to other techniques such as ink-jet printers, lithography, etc., makes μCP-fabricated arrays very attractive (Lin et al. 2001). Using direct μCP of proteins, we were able to develop microarray immunoassays for antibodies (Nichkova et al. 2006) and biomarkers of pyrethroid exposure (Nichkova et al. 2005) based on Eu:Gd_2O_3 nanoparticles as fluorescent reporters.

Immunoassays are used as screening tools for the assessment of human exposure to a variety of agrochemicals (Jaeger, Jones, and Hammock 1998; Shan et al. 2004). Because of the prevalent use of pesticide mixtures, people

have simultaneous exposure to multiple pesticides that may cause enhanced toxicity via synergistic effects. Multiplexed assays are needed for occupational toxicology, risk assessment, and epidemiological studies. In this work, we focus on the detection of biomarkers of exposure to two major classes of compounds: pyrethroid insecticides and triazine herbicides. Pyrethroids are widely used in vector control, forestry, households, agriculture, and horticulture. As major pyrethroids contain the phenoxybenzyl group and phenoxybenzoic acid (PBA) is a common metabolite form or intermediate, PBA can be used as a generic biomarker of human exposure to most pyrethroids (see Fig. 1) (Shan et al. 2004). Atrazine is one of the most widely used herbicides in the US, and despite its low acute toxicity to humans, it may pose a health risk to humans, especially to agricultural workers though occupational exposure. The main urinary metabolite, atrazine-mercapturate (N-acetyl cysteine derivative of atrazine) (Lucas et al. 1993) has been suggested as relevant biomarker for atrazine exposure in humans (see Fig. 1) (Buchholz et al. 1999).

The aim of this work is to apply QDs as fluorescent labels in multiplexed micro-immunoassays for detection of PBA and AM as biomarkers of exposure to pyrethroids and atrazine. Microarrays are prepared with μCP and confocal microscopy is used for fluorescence measurements.

EXPERIMENTAL

Chemicals and Materials

Atrazine mercapturic acid (AM) [N-acetyl-S-{4-(ethylamino)-6-[(1-methylethylamino]-1,3,5-triazin-2-yl}-L-cysteine] was prepared in our

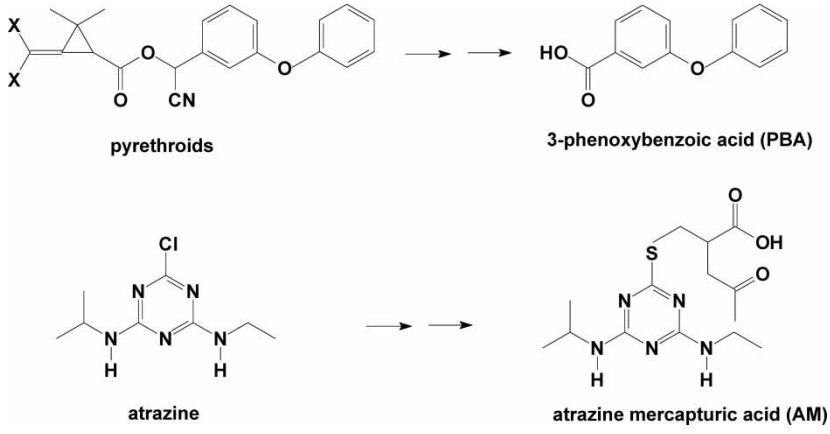

Figure 1. Chemical structures of targeted agrochemicals (pyrethroids and atrazine) and their biomarkers of exposure (PBA and AM).

laboratory previously (Lucas et al. 1993). 3-Phenoxybenzoic acid (PBA) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, Missouri). The preparation of the coating antigen BSA-PBA, and the anti-PBA specific polyclonal rabbit antibody (As 294) (Shan et al. 2004), and the coating antigen BSA-AM (7-BSA) and the polyclonal sheep anti-AM (As 266) (Jaeger, Jones, and Hammock 1998) have been reported previously. The IgG fractions from As294 and As226 were obtained by protein A purification using Affi-gel protein A MAPS® II kit (Bio-Rad Laboratories, California). IgG226 was biotinylated using sulfo-NHS-biotin (Pierce, Illinois). Goat anti-rabbit IgG F(ab')2 conjugated to Birch Yellow EviTag (anti-rabbit QD580, 5 mg/ml protein concentration) and streptavidin conjugated to Maple Red EviTag (SA-QD620, 5 mg/ml protein concentration) were obtained form Antibodies Inc. (Davis, California). Clay Adams cover glass from Fisher Scientific (Pittsburgh, Pennsylvania) was used as a substrate for microcontact printing of proteins and fluorescence characterization and a *n*-doped Si wafer was used for SEM characterization. The polydimethylsiloxane (PDMS) stamp was prepared according to the literature method (Kumar and Whitesides 1993) using Sylgard 184 silicon elastomer (Dow Corning, Midland, Michigan). The stamp used in the microcontact printing had a patterned matrix of 10×10 μm strips.

Instrumentation

Fluorescent images were acquired with a Leica TCS-SP Laser Scanning Confocal Microscope equipped with UV, Argon, Krypton, and Helium-Neon lasers for excitation and photomultiplier tubes (PMTs) for detection. Leica Confocal Software LCS Lite 2.0 was used for image acquisition. A UV laser beam was used for the excitation of QD580 and 620. For SEM characterization a FEI XL30-SFEG Scanning Electron Microscope was used.

Multiplexed Fluorescence Microimmunoassay for PBA and AM

Substrate Preparation

Cover glass was used as a solid substrate for microcontact printing of the coating antigen BSA-PBA. Prior to use, the glass substrate was cleaned by immersion in a solution of H_2SO_4:H_2O_2 (70:30 v/v) for 2 h and then washed extensively in running deionized water. Then the glass substrate was thoroughly washed in an ultrasonic bath in ethanol and deionized water for 10 min each. Finally, it was dried under a blowing nitrogen stream. The PDMS stamp was washed by sonication in ethanol (3×10 min), dried under nitrogen and exposed to the solution of the inking protein mixture (BSA-PBA and BSA in PBS in different ratios with 50 μg ml^{-1} total protein concentration) for 40 min. Excess solution was removed, and the stamp was dried under nitrogen. After inking, the stamp was brought into

contact with the glass substrate and a very small amount of force was applied to make a good contact between both surfaces. The BSA-PBA printing stage is schematically presented in Fig. 2A. Printed slides were incubated with the 2 mg ml^{-1} BSA for blocking in the case of single analyte assays. If substrates were prepared for multiplex analysis, they were incubated with the second coating antigen (BSA-AM and BSA in PBS in different ratios with 2 mg ml^{-1} total protein concentration) for 1 h and rinsed with water (Fig. 2B).

Competitive Micro-Immunoassay

The substrates were incubated for 1 h with the specific antibodies (50 $\mu g\ ml^{-1}$ IgG294 and 20 $\mu g\ ml^{-1}$ biotinylated IgG226) in 0.5% BSA/PBS, allowing the specific interaction between antibody and antigen to occur (Fig. 2C). In the competitive assays, PBA and AM at 100 $\mu g\ l^{-1}$ concentration were added to the antibody solution. After washing with buffer, the substrate was incubated for 1 h with anti-rabbit QD580 (1/50 dilution in 0.5% BSA/PBS) and SA-QD620 (1/30 dilution in 0.5% BSA/PBS) (Fig. 2D). After the interaction took place, the substrate was rinsed with buffer, water, dried under nitrogen, and scanned for fluorescence evaluation.

Scanning and Evaluation

A UV laser beam was used for the excitation of the two QDs used in this work. The detection wavelength ranges of the two PMTs were independently adjusted to 625–635 nm for QD 620 and 565–575 nm for QD 580. The gain of each PMT was individually adjusted to obtain similar fluorescence intensities from the QD 620 labeled strips and the QD 580 labeled strips. The average of five strips was used for evaluation of signal intensity. Each experiment was performed in duplicate.

RESULTS AND DISCUSSION

With the aim to develop multiplexed immunoassays for biomarkers for exposure we have chosen two targets: PBA and AM (see Fig. 1). The specific antibodies against those analytes are rabbit and sheep polyclonal antibodies, respectively. We expected low cross-reactivity in the multiplexed system. Initially we tested anti-rabbit and anti-sheep secondary fragments labeled with QDots, but the S/N ratio obtained for the anti-sheep system was very low. Therefore, we biotinylated the sheep antibody and used biotin-streptavidin amplification detection. As reporters we have selected QDot 580 and QDot 620 because under UV excitation they have well defined separate fluorescence emission peaks centered at 570 and 630 nm, respectively (see Fig. 3).

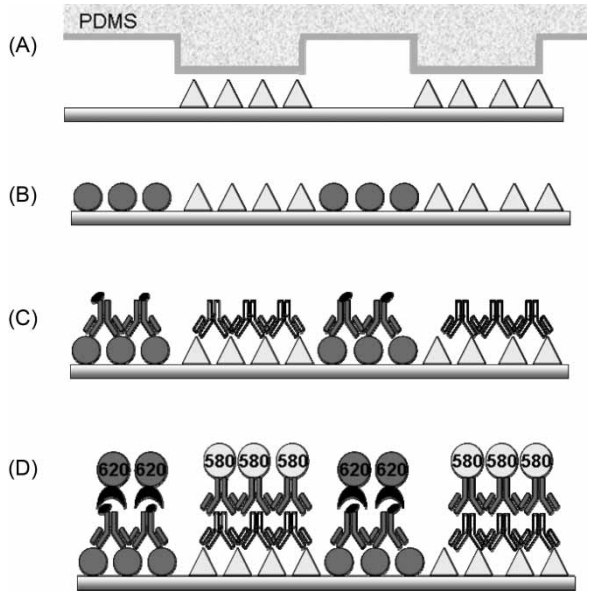

Figure 2. Scheme of the multiplexed immunoassay for PBA and AM. (A) microcontact printing of the coating antigen (BSA-PBA); (B) immobilization of the coating antigen (BSA-AM); (C) immunoreaction with anti-PBA antibody and biotinylated anti-AM antibody; (D) incubation of the micro-patterns with the secondary immunoreagents (anti-rabbit QDot 580 and streptavidin-Qdot 620).

Single Analyte Microimmunoassay

Initially we tested the QD detection system in a single analyte format. Glass substrates patterned with BSA-PBA (or AM) and blocked with BSA were

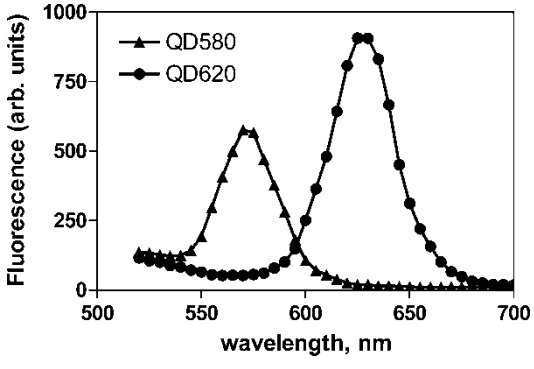

Figure 3. Fluorescent spectra of anti-rabbit QDot 580 and streptavidin-Qdot 620 (excitation at 350 nm) recorded in Spectramax M2 microplate reader.

used and different concentrations of secondary reagents were tested. Typical fluorescent image and the corresponding line intensity profile of the obtained micropatterns for the PBA assays are shown in Fig. 4A and B. A series of alternating bright (green) and dark strips can be observed. The actual width of the strips (10 μm) is defined by the PDMS stamp used. The green strips correspond to the QDots 580 specifically bound to the printed BSA-PBA and the dark strips correspond to the BSA-blocked spacings between them. The average signal-to-noise ratio (S/N), i.e. specific intensity/background intensity of $\sim$10 demonstrates successful immunoreaction with very low nonspecific binding. Figure 4C represents the effect of the coating antigen concentration (BSA-PBA) used in the printing process on the fluorescence signal of QD 580. Saturation typical for this type of

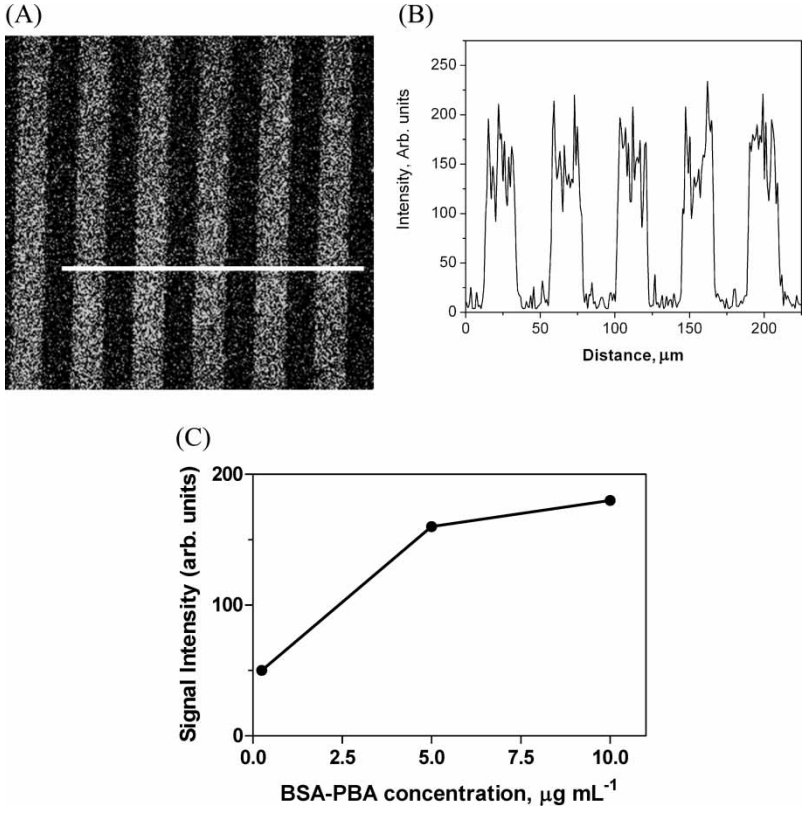

Figure 4. Single analyte micro-immunoassay for PBA (A) Fluorescence image of QD 580 specifically bound to anti-PBA rabbit IgG bound to microcontact printed BSA-PBA antigen; (B) Intensity profile corresponding to line shown in (A); (C) Fluorescent intensity of QD 580 as a function of the concentration of the microcontact-printed BSA-PBA coating antigen.

immunoreactions is observed. We have chosen a concentration of 4 $\mu g\,ml^{-1}$ for the competitive assay. Similarly, we have tested the experimental conditions for the AM immunoassay in a single analyte format (data not shown). Micropatterns with red strips corresponding to SA-QDot 620 bound to the biotinylated anti-AM antibody recognizing the printed BSA-AM were obtained.

SEM Characterization of the Microarrays with QD

The microarrays were characterized by scanning electron microscopy (SEM) for more detailed studies at single nanocrystal level. For this purpose, the micro-patterns were prepared on a silicon wafer instead of glass slide following the procedure described above and keeping all other experimental conditions identical. The use of the silicon wafer assured a perfectly flat surface and conductivity of the substrate—conditions required for the SEM studies. The SEM image presented in Fig. 5 illustrates a segment of a strip formed by the specifically bound anti-rabbit QD 620 to anti-PBA rabbit IgG bound to microcontact printed BSA-PBA coating antigen. The wafer was passivated by BSA blocking. The QDots can be individually distinguished as white spots on the dark background. It can be seen that the distribution of the QDs is not perfectly uniform and the QDs are not densely packed within the strip. A similar effect was observed by other authors when immobilizing polystyrene beads through specific avidin-biotin binding (Andersson et al. 2002) and also when using lanthanide oxide particles as labels in similar configurations (Nichkova et al. 2006). We believe that this is mainly due to steric hindrance and limited diffusion of the QDs near the substrate during the incubation. In spite of that, the QDs are arranged into a good match with the printed patterns. Only a few individual QDs can be observed in the non-printed areas blocked with BSA, thus showing very low non-specific binding.

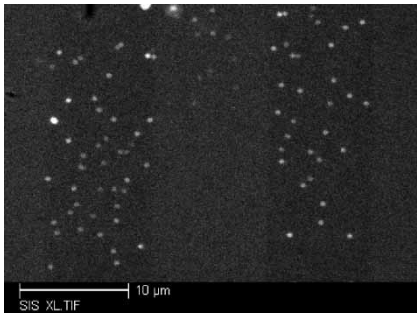

Figure 5. Scanning Electron Microscopy (SEM) image of specifically bound anti-rabbit QD 580 to anti-PBA rabbit IgG bound to micro-contact printed BSA-PBA coating antigen (10 × 10 μm line patterns). BSA is used for blocking the non printed areas.

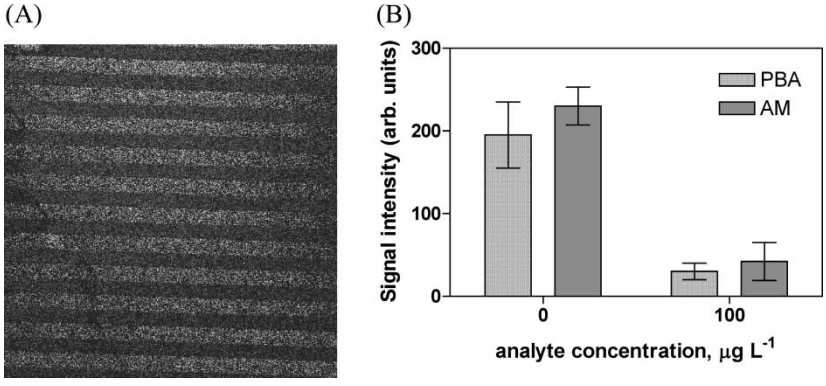

Figure 6. Multiplexed immunoassay for PBA and AM. (A). Confocal microscope fluorescent image corresponding to QD 580 (PBA) and QD 620 (AM); (B). Competitive micro-immunoassay (signal inhibition in presence of both analytes).

Multiplexed Fluorescence Microimmunoassay for PBA and AM

The multiplexed micro-immunoassay was performed with glass substrates containing strips formed by the two coating antigens (BSA-PBA and BSA-AM). Fig. 6A represents the fluorescence image obtained for the multiplexed assay at zero analyte concentration. Strips with alternating green and red colors corresponding to QD 580 and QD 620 can be observed. The difference in the spectral emission of the two QDs enables their simultaneous detection without cross-talk. In a mixture of the analytes (PBA and AM), the fluorescence signal was inhibited because fewer amounts of specific antibodies bind to the substrate resulting in less binding of the secondary reagents (QDs) (see Fig. 6B). Further optimization of the quantitative measurements would require the incorporation of internal fluorescence calibration of the detection system (Nichkova et al. 2005) and optimization of the sensitivity of the multiplexed immunoassay. Using europium oxide nanoparticles as labels in similar configuration, we have obtained sensitivity for PBA comparable to that of the ELISA (Nichkova et al. 2005).

CONCLUSIONS

Quantum dots can be used as suitable labels for imaging of microarrays for the detection of small molecules (biomarkers of pesticide exposure). Their photo stability permits prolonged excitation for image observation and optimization. The spectral properties of quantum dots allow the multi-analyte detection of the targets. The same approach could be successfully applied to the detection of proteins and DNA with different bioanalytical applications.

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