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Journal
Journal of Polymer Science Part A Polymer Chemistry, 53(2)

ISSN
0887-624X

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Publication Date
2015-01-15

DOI
10.1002/pola.27376

Peer reviewed

A Turn-Off Fluorescent Substrate for Horseradish Peroxidase Improves the Sensitivity of ELISAs

This manuscript is dedicated to Prof. Jean Fréchet on the occasion of his 70th birthday and to his extraordinary contributions to polymer science.

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Received 4 June 2014; accepted 20 August 2014; published online 00 Month 2014
DOI: 10.1002/pola.27376

KEYWORDS: biomaterials; chlamydia; cyanine; diagnostics; ELISA; enzymes; liposome; nanotechnology; ultrasensitive

INTRODUCTION There is a great need for developing low cost diagnostics that can be used in the developing world. Enzyme-linked immunosorbant assays (ELISAs) have great potential as a low cost diagnostic tool because they are widely used in the developing world, however, their low sensitivity has limited their application. In this report, we demonstrate that a new horseradish peroxidase (HRP) turn-off fluorescent substrate termed clics (cyanine liposome complexes) can enhance the sensitivity of conventional ELISA kits by 32-fold, and could detect chlamydial infection in patients. The clics are highly sensitivity, cost-effective, and compatible with commercial ELISA kits, and therefore they can impact numerous areas of diagnostics in the developing world.

ELISA is widely used for detection of diseases in resource poor settings, because they are easy to use and do not require specialized instruments. However, the detection sensitivity of ELISAs is in the nanomolar/picomolar range, and therefore they cannot be used for early detection of disease biomarkers, which are frequently present at the femtomolar range.[1–5] The low sensitivity of ELISAs is attributed to absorbance based substrates of HRP, such as tetramethylbenzidine (TMB), whose absorption detection limits are in micromolar range. Although, highly sensitive fluorescent HRP probes, which have detection limits in the nanomolar range, have been developed, they have not been utilized in resource-poor settings because of their instability.[2,6,7] Therefore, there is a great need to develop new stable and fluorescent HRP substrates for applications in the developing world.

In this report, we present a new ultrasensitive and stable HRP substrate, termed clics, composed of cyanine dye and hydroxyl radical stabilizers intercalated in lipid bilayers, which enhance the sensitivity of conventional ELISAs. Clics enhance the sensitivity of commercial ELISA kits because they are fluorescent reporters of HRP. Figure 1 shows the mechanism by which clics enhance the sensitivity of commercial ELISA kits. Hydroxyl radicals generated by HRP diffuse through the lipid bilayer and degrade the cyanine dye IR783 by one-electron oxidation, followed by cleavage of the polymethine chain, thereby quenching their fluorescence.[7] Although, turn-off fluorescent techniques have the potential for false positives, we anticipate that this can be addressed by performing calibration curves, simultaneously with the patient sample. There are numerous applications of highly sensitive clic-based ELISAs, and we utilized them to enhance the sensitivity of ELISAs for detecting *C. trachomatis* infection, a sexually transmitted disease prevalent in resource poor areas of the world.[8] Clics are easily incorporated in clinically used commercial ELISA kits and, therefore, they can have a great impact in all fields of medicine.

Clics are designed to detect HRP via hydroxyl radical mediated degradation of cyanine dyes. We performed experiments to determine whether IR783 is a substrate for HRP. IR783 was resuspended in phosphate buffer saline (PBS) and added to the wells of a 96-well plate containing 1–2 dilutions of HRP with 24×10^{-15} M as the highest concentration and PBS as the negative control. Figure 2(A) demonstrates that the reduction in fluorescence of IR783 is directly proportional to the HRP concentration and IR783 can detect 24×10^{-15} M levels of HRP, thereby enhancing the detection sensitivity of HRP by 2 orders of magnitude as compared to TMB. However, the R^2 of IR783 was only 0.83, which we speculate was due to its aggregation or photo- and chemical degradation.[9]

Abhinav P. Acharya and Payel Sen contributed equally to this work.

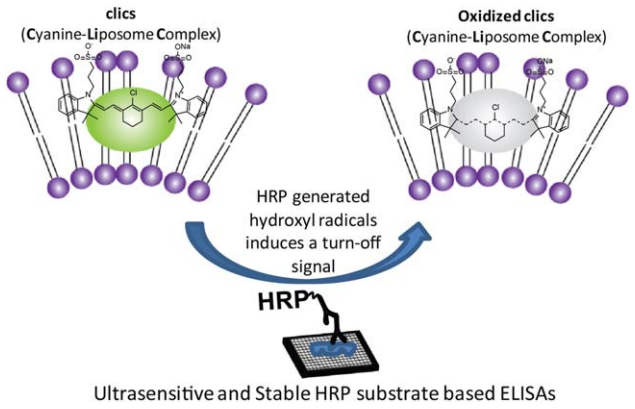

FIGURE 1 Cyanine-liposome complexes (clics) are a new family of fluorescent probes for HRP, which can be combined with commercial ELISA kits to enhance their sensitivity. Clics are composed of a cyanine dye intercalated in a lipid bilayer. HRP specific oxidation of the IR783 cyanine dye leads to the decrease in fluorescence, which is directly proportional to the concentration of HRP. Clics are easily incorporated in conventional ELISA kits and commercial plate readers.

We performed experiments to determine whether the stability of IR783 could be improved by encapsulating the dye in a lipid bilayer. Dimyristoyl phosphotidylcholine (DMPC) bilayers containing IR783 and 4-hydroxy-4-biphenyl carboxylic acid (a hydroxyl radical stabilizer) were generated (termed clics), and their sensitivity toward HRP was investigated. Figure 2(A) demonstrates that clics increase the sensitivity of IR783 for HRP by 25-fold and also improve the accuracy, having an R^2 of 0.98. In addition, clics are 3 orders of magnitude more sensitive to HRP than commercially available TMB [Fig. 2(B)]. In addition, we verified that HRP generated hydroxyl radicals were degrading IR783 via mass spectrometry, and we observed IR783 degradation products at 296.0959 and 248.9603 amu, (observed by electrospray ionization in a negative-ion mode (QB3/Chemistry Mass Spectrometry Facility at the University of California, Berkeley)).

There are numerous applications of clic-based ELISAs, given their high sensitivity, stability, and low cost, particularly for screening of diseases endemic in resource poor countries. We utilized clic-based ELISAs for detecting *C. trachomatis* infections, a sexually transmitted pathogen that affects 300–500 million people worldwide.[10,11] This infection can be asymptomic and persistent, and needs to be detected early to prevent its spread. Although, chlamydia infections can be diagnosed by nucleic acid amplification tests (NAATs), these tests are not widely used due to their high costs, and requirement of specialized instruments and skilled labor.[12] Individuals with chlamydiae often generate antibodies against the chlamydial heat shock protein HSP60, and therefore, chlamydial HSP60 antibody is a good diagnostic biomarker for screening patients.[13,14] However, chlamydial *HSP60* antibody ELISAs are not ultrasensitive for early detection of infection and, therefore, there is a great need for improving their sensitivity.

We constructed ELISAs to detect chlamydial *HSP60* antibodies, by adsorbing *cHSP60* protein in the wells of a 96-well plate. Different dilutions of chlamydial *HSP60* monoclonal antibody was then incubated in the wells of these plates and the antibodies bound to chlamydial *HSP60* protein was detected using an HRP labeled secondary antibody, and clics or TMB substrate. Figure 3(A,B) demonstrate that TMB can detect 5 ng/mL chlamydial *HSP60* antibodies (P-value < 0.05), whereas clics can detect 0.156 ng/mL chlamydial *HSP60* antibodies (P-value < 0.05), thereby providing a 32-fold enhancement in the detection limit.

Next, in order to utilize the high sensitivity of clics in detecting chlamydial infections, we designed a preliminary blinded study where serum of patients was diluted up to 100-fold to represent early infection levels of antibodies in the blood. The serum of patients positive or negative for infection were identified using clinically used NAAT and these serum samples were chosen to be tested via clic- and TMB-based ELISAs. In order to detect

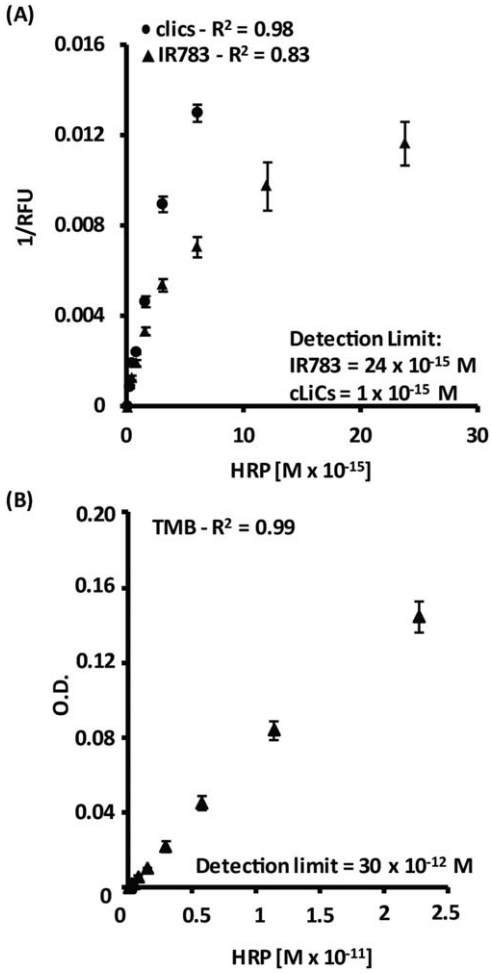

FIGURE 2 Clics are substrates for HRP and can detect 1 femtomolar of soluble HRP. A: IR783 detects 24 femtomolar levels of soluble HRP in a 96-well plate. Clics detect 1 femtomolar levels or 100,000 molecules of soluble HRP in a 96-well plate. B: TMB detects 30 picomolar levels of soluble HRP in a 96-well plate.

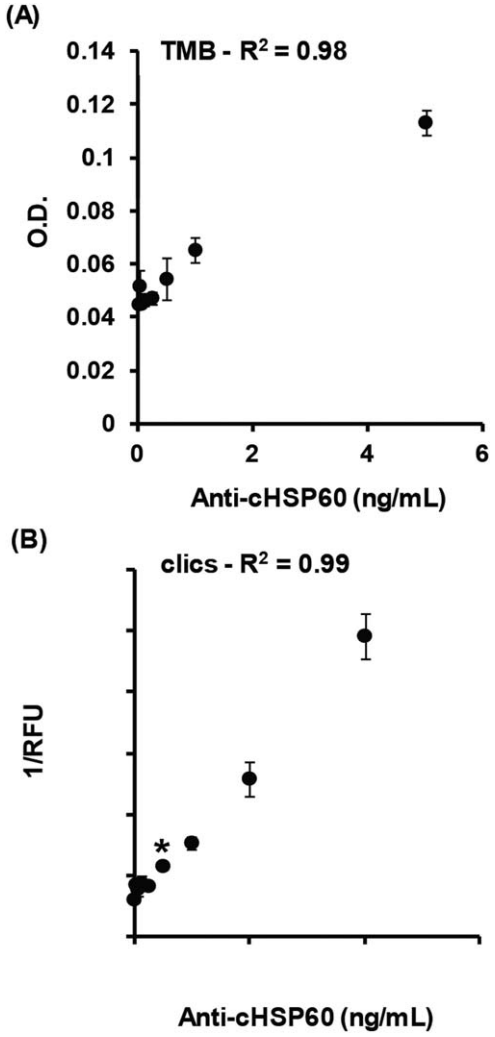

FIGURE 3 Clic-based ELISAs detect anti-chlamydial HSP60 antibodies and are 32-fold more sensitive than TMB-based ELISAs. A: TMB-based ELISAs were generated by adsorbing the recombinantly expressed chlamydial *HSP60* protein on a 96-well plate, applying diluted patient serum and detecting anti-*cHSP60* via conventional TMB substrate. TMB-based ELISAs were able to detect anti-*cHSP60* (*$P < 0.05$) at 5 ng/mL. B: Clic-based ELISAs were generated in the same manner as TMB-based ELISAs except, clics were added as the HRP substrate instead of TMB. Clic-based anti-*cHSP60* ELISAs could detect 0.156 ng/mL of anti-*cHSP60* (*$P < 0.05$) and, therefore, enhance the sensitivity of ELISAs by 32-fold.

chlamydial antibody responses, clic- or TMB-based ELISAs were performed on human serum samples diluted 100-fold in PBS for detecting chlamydial *HSP60* IgM antibodies. Figure 4(A,B) demonstrate that clic-based ELISAs can detect chlamydial HSP60 IgM antibodies in infected patients, at 100-fold dilution of serum samples, whereas, TMB-based ELISAs were not able to detect positively infected patient at 10- or 100-fold diluted samples.

CONCLUSIONS

In conclusion, we have demonstrated that clics are excellent HRP substrates and can detect as few as 1 femtomolar soluble HRP. Clics increase the sensitivity of ELISA kits by 32-fold and since, clic-based ELISAs can be easily used with commercial ELISA kits and plate reader, they should be able to enhance the sensitivity of a large number of commercial ELISA kits, enabling their rapid use by the molecular biology and clinical laboratories. Importantly, clics are stable at room temperature and, therefore, clic-based ELISAs can be utilized in resource poor settings to detect chlamydial antibodies in human serum samples.

EXPERIMENTAL

Ethics Statement

The patient sera used was de-identified with no trace to patient names and thus is not considered human subject's research.

Synthesis of Clics

Clics were developed by mixing IR783 dyes (Sigma Aldrich, Milwaukee, WI) with DMPC bilayer lipid structures (Avanti Polar Lipids, Alabaster, Alabama). About 500 μL of 1 mg/mL of 4-hydroxy-4-biphenyl carboxylic acid (Sigma Aldrich, Milwaukee, WI) in methanol (Sigma Aldrich, Milwaukee, WI), 100 μL of 1 mg/mL of IR783 in dichloromethane (DCM), and 200 μL of 1 mg/mL DMPC in chloroform (Sigma Aldrich, Milwaukee, WI) were added to 10 mL of dichloromethane (Sigma Aldrich, Milwaukee, WI) in a 100-mL round bottom flask. The solution was sonicated using a sonicating probe (Barson Corporation). The dichloromethane was evaporated

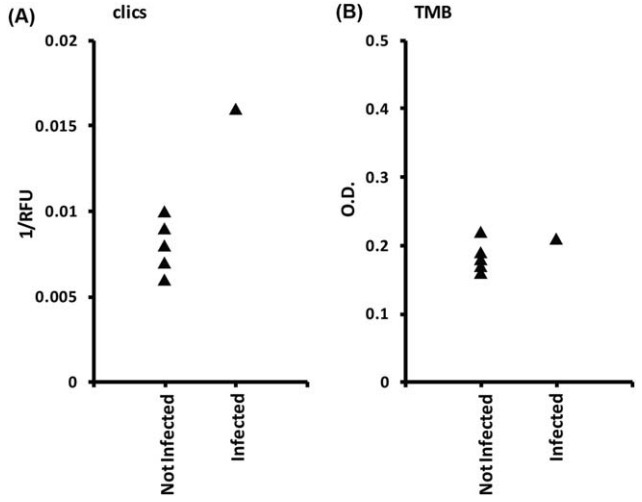

FIGURE 4 Clics-based ELISAs enhance the sensitivity of detection of anti-chlamydia HSP60 IgM antibodies in patient serum. A: Clic-based ELISAs were performed to detect anti-chlamydia IgM antibodies present in 100-fold diluted human serum obtained from patients with and without *C. trachomatis* infection. Clic-based ELISAs were able to identify positively infected patient from negative controls. B: TMB-based ELISAs were performed to detect anti-chlamydia IgM antibodies present in 100-fold diluted serum obtained from patients with and without *C. trachomatis* infection. TMB-based ELISAs were not able to identify positively infected patients from negative controls.

using the rotary evaporator at room temperature to completion. Next, 10 mL PBS (VWR, Batavia, IL) was added to the flask. This solution was then stored at 4 °C and utilized for all the experiments.

IR783 Sensitivity to HRP

We investigated whether IR783 could be utilized as the substrates of HRP. About 300 μg of IR783 resuspended in 50 mL PBS was diluted 1:1 with slow TMB solution (Thermo Scientific) and was used immediately as the HRP substrate. Eppendorf tubes and pipette tips were made resistant to protein adsorption by incubating them in 1 mg/mL solution of pluronic 108 (Sigma Aldrich) for 1 min. About 1–2 HRP dilutions were generated in these eppendorf tubes with the highest concentration of 24×10^{-15} M, and 50 μL of the HRP solutions was added to the wells of a 96-well plate. About 50 μL of IR783 substrate was added to the wells, and fluorescence ($E_x/E_m = 780/820$ nm) was recorded using a plate reader (Tecan i4) after 5 min. Reciprocal of the fluorescence was obtained and plotted against the concentration of HRP.

Clics Sensitivity to HRP

We investigated whether clics could enhance the stability of IR783 and increase the sensitivity toward HRP. Dilutions of HRP solutions were generated with the highest concentration of 6×10^{-15} M. About 50 μL of HRP solution was added to the wells of a 96-well plate. About 50 μL of clics in PBS (Sigma Aldrich, Milwaukee, WI) was added to the wells containing HRP, and fluorescence ($E_x/E_m = 780/820$ nm) was recorded using a plate reader after 5 min.

Chlamydial HSP60 Expression and Purification

C. trachomatis strain E genomic DNA was amplified by polymerase chain reaction (PCR) using primers specific for the chlamydia heat shock protein 60 (*cHSP60*) gene that contained restriction enzyme sites Nhe1 and Xho1. The PCR product was cloned into the pCR-Blunt Vector using the Zero Blunt PCR Cloning kit (Life Technologies) and then transformed into One shot TOP10 Chemically Competent *E. coli* supplied with the kit. The recombinant plasmid was isolated using a miniprep kit (GenJet; Thermo Scientific). Double restriction enzyme digest using Nhe1 and Xho1 was performed at 37 °C for 2 h to cut out the insert. The expression vector pET 28a with Histidine Tag at the C terminus was also digested by enzymes Nhe1 and Xho1 at 37 °C for 2 h. Ligation was performed overnight at 16 °C and the product was transformed into One Shot TOP10 chemically competent *E. coli*. The plasmid pET28-HSP60 was isolated by the miniprep kit and then retransformed into the BL21De3 expression cell line. Protein induction was performed using 0.5 mM IPTG at 37 °C and testing the optical density (O.D.) of the cells for 3 h until and O.D. of 0.6 was reached. The induction was analyzed by running the protein on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) gel and then staining with coomassie. Protein purification was performed under denaturing conditions using the PROBOND purification kit (Invitrogen) at room temperature. Western Blot using anti-His tag antibody (Invitrogen) was performed to confirm the recombinant chlamydial HSP60 protein in the final elution (data not shown).

Clic-based ELISAs for Detecting Anti-chlamydial HSP60

We investigated whether clics can increase the accuracy of detection of HSP60 using anti-chlamydial HSP60 monoclonal antibodies (Santa Cruz Biotechnology). About 1 μg/mL of rHSP60 in a 50 μL volume was incubated in the wells of a 96-well plate (Greiner bio-one) overnight at 4 °C. The wells were then washed three times with 300 μL of wash buffer made of 0.05% tween 20 (Sigma Aldrich) in PBS (VWR). 50 μL of a solution containing 1% bovine serum albumin (BSA) (Sigma Aldrich) and 0.05% tween were added to the wells and incubated for 1 h to block non-specific adsorption. The wells were then washed three times with 300 μL of wash buffer. Serial 1–2 dilutions of 5 ng/mL anti-chlamydia HSP60 monoclonal antibodies were performed in 1% BSA and 0.05% tween 20. 50 μL of the serially diluted antibodies were then added to the wells and incubated for 1 h. The wells were washed three times with 300 μL of wash buffer, and 50 μL of 1:1000 diluted, HRP linked anti-mouse secondary antibody (Santa Cruz Biotechnology) in 1% BSA and 0.05% tween was incubated in the wells for 1 h. The wells were again washed three times with 300 μL of wash buffer, and 100 μL of TMB or clics substrate was added to the wells. Absorbance or fluorescence reading was obtained using the Tecan plate reader.

Clic-based ELISAs for Detecting Anti-chlamydial HSP60 in Human Serum

Six human de-identified serum samples from patients at high risk for sexually transmitted diseases were diluted at 100-fold with PBS. About 50 μL of chlamydial HSP60 (1 μg/mL) was incubated overnight at 4 °C in a 96-well plate. On the following day, washes were done with 300 μL of washing buffer (PBS with 0.05% tween-20), and the protein was blocked with 100 μL of 1% BSA in PBS for 1 h. The wells were washed with washing buffer, and 50 μL of the diluted human serum made in PBS was incubated in the wells for another hour. The wells were washed three times with the washing buffer and then incubated with 50 μL of anti-human HRP linked IgM at 1:5000 dilution (Millipore) for 1 h. The wells were then washed with washing buffer three times and then twice with PBS. About 100 μL of TMB or clics substrate was then added to analyze the absorbance or fluorescence using the Tecan plate reader.

Statistical Analysis

Statistical analyses were performed using student's *t*-test, and P-values for each experiment were determined. Statistically significant data ($P < 0.05$) are depicted using the "*" symbol.

ACKNOWLEDGMENTS

The authors declare no competing financial interest. This work was supported by the following grants to NM from the NIH, 1R01HL096796-02, NIH: U01 268201000043C-0-0-1, NIH:

1R01HL090601-02, NIH: 1R21AI098799-01, 7UM1AI068636-07, NIH RO1 AI088023-03, and the following grants to DD from the NIH: R01 AI098843 and R44 AI084206.

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