

# Use of Highly Porous Polymer in a Diagnostic Assay

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Array-based methods have made substantial contributions to diagnostic assays. In order to accommodate the need for multiplex testing, single-analyte tests have evolved into array-based formats.<sup>1</sup> This bead-based technology has provided investigators with a new approach to simultaneously measure multiple analytes in biological samples.<sup>2</sup> Advantages of bead-based assays include multiple analyte evaluation in a single sample, minimal sample volume, rapid evaluation of multiple samples in a plate form, and direct comparison with existing assays.

However, there are limitations to conventional porous polymer bead materials that may preclude optimum binding of antigen-antibody complexes and limit mass transfer. These limitations include irregularity of the pores, their relatively small size (angstrom dimensions), and their termination within the polymer body.<sup>3</sup> It is speculated that binding of antigen-antibody complexes may be less efficient in these conventional materials due to steric hindrance caused by pore irregularity. Furthermore, it is speculated that mass transfer characteristics may be less than optimum since pores are not fully interconnected.

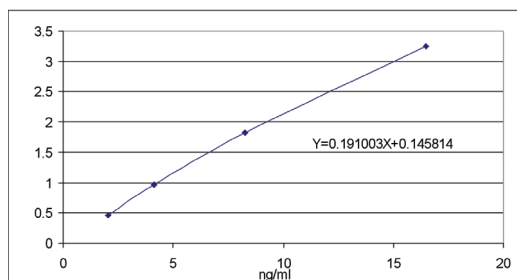


Figure 1 Standard curve for the anti-horse IgG-PO. Anti-horse IgG-PO was incubated with urea-H<sub>2</sub>O<sub>2</sub> and OPD substrate solution at different concentrations for 40 min. Developed red solution was read at 416 nm.

A spherical high internal phase emulsion polymeric matrix featuring characteristics that may avoid the limitations of conventional porous polymers has been developed. The underlying technology for Cavilink™ porous polymers (Polygenetics, Inc., Mountain View, CA)<sup>4</sup> was developed by Unilever Plc. (London, U.K.).<sup>5</sup> In the authors' work, a cross-linked polystyrene matrix possessing high porosity (up to 90% by volume) was utilized. The Cavilink spheres contain very large cavities (with diameters of micrometer dimensions) and high absorbency.<sup>6</sup> Furthermore, these large cavities are fully interconnected, thereby allowing free ingress and egress of reactive solutes. These important characteristics prompted an investigation of their utility in diagnostic assay systems.

## Materials and methods

The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO): anti-horse IgG with peroxidase conjugated (anti-horse IgG-PO at 3.3 mg/mL), anti-horse IgG with alkaline phosphatase conjugated (anti-horse IgG-ALP at 1.0 mg/mL), alkaline phosphatase working substrate kit containing bromo-chloro-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT), peroxidase substrate kit containing urea-hydrogen peroxide and orthophenyldiamine (OPD) tablets, and phosphate-buffered saline (PBS). Cavilink

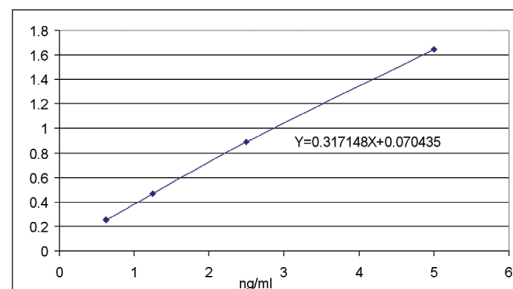


Figure 2 Standard curve for the anti-horse IgG-ALP. Anti-horse IgG-ALP was incubated with BCIP and NBT substrate solution at different concentrations for 30 min. Developed blue color solution was read at 614 nm.

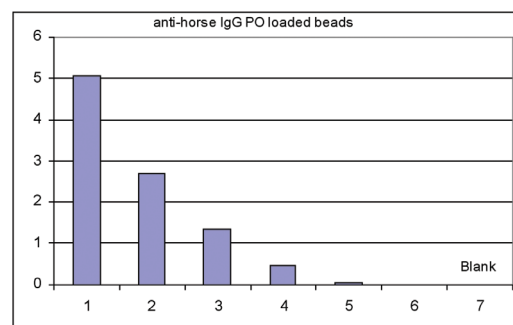
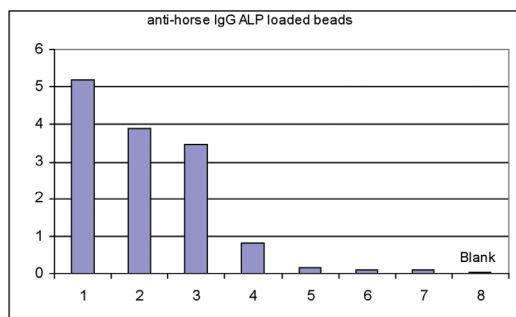


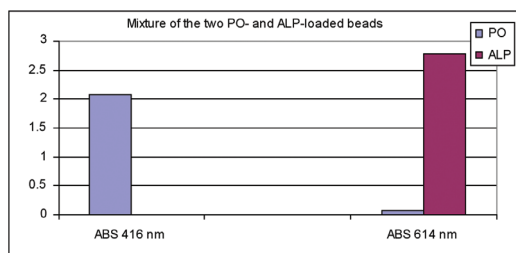
Figure 3 Detection of anti-horse IgG-PO. Increase in the detection of anti-horse IgG-PO in the beads as a function of the level of loading in the beads. Anti-horse IgG-PO was loaded into the beads and detected as described in the text.

polymer beads with diameters in the 250–300 μm range were produced by Polygenetics. A Helios gamma spectrophotometer (Thermo Spectronics, Rochester, NY) was used for absorbance measurements.

Three milliliters of either anti-horse IgG-PO or anti-horse IgG-ALP was added to 0.05 g beads by rotating for 1 hr at room temperature. Anti-horse IgG-PO was added to the beads at the range of 66 ng/mL to 0.33 mg/mL in PBS. Anti-horse IgG-ALP was added at the range of 0.2 ng/mL to 20 μg/mL in PBS. After mixing, the beads



**Figure 4** Detection of anti-horse IgG-ALP. Increase in the detection of anti-horse IgG-ALP in the beads as a function of the level of loading in the beads. Anti-horse IgG-ALP was loaded into the beads and detected as described in the text.



**Figure 5** Interference study: interference between PO or ALP enzyme system, in a mixture of two types of loaded beads. Beads loaded with anti-horse IgG-PO were mixed with beads loaded with anti-horse IgG-ALP and levels of two enzymes were determined using either of the two substrate solutions.

were filtered and air-dried overnight. Substrate solution for PO, conjugated to anti-horse IgG, was prepared by dissolving one urea-hydrogen peroxide tablet and one OPD tablet in 20 mL H<sub>2</sub>O. This resulted in 0.4 mg/mL of each component. Substrate solution for ALP, conjugated to anti-horse IgG, was prepared by mixing BCIP solution and NBT solution at a 1:1 ratio. For each case, 3 mL of each substrate solution was added to 0.005 g of the loaded bead. A mixture of beads and corresponding substrate solution was incubated at room temperature for 2–30 min. After incubation, the mixture of beads and the developed colored solution was filtered through a fiberglass filter. The absorbance of red color developed due to PO reaction was read at 416 nm and that of blue color developed due to ALP reaction was read at 614 nm.

## Results and discussion

Figures 1 and 2 show the standard curves for anti-horse IgG-PO and anti-horse IgG-ALP, respectively. Calculations have shown that the maximum level of anti-horse IgG-PO loaded was 19.48 µg/g of beads and that of anti-horse IgG-ALP was 12.01 µg/g of beads.

Furthermore, calculations showed that anti-horse IgG-PO can be detected as low as 3.79 µg/g of beads and anti-horse IgG-ALP can be detected as low as 1.48 µg/g of beads. Using the value of 42,462 beads/cc, the maximum loading of anti-horse IgG-PO onto a bead was 22.94 pg/bead and for anti-horse IgG-ALP was 14.14 pg/bead. Also, it has been demonstrated that the presence of anti-horse IgG-PO at as low as 4.46 pg/bead is detected and that of anti-horse IgG-ALP is as low as 1.74 pg/bead. These two values indicate the sensitivity of the assay for IgG-PO and IgG-ALP, loaded onto the beads.

Reactions of either PO or ALP in the presence of their substrate solutions (i.e., OPD or NBT solutions, respectively) take place on each loaded bead. This is demonstrated in Figures 3 and 4. As shown, while the absorbance of the respective substrate solutions increased due to an increase in the amount of the antibody loaded onto the beads (either anti-horse IgG-PO or IgG-ALP), the beads with no antibody (i.e., blank) showed absorbance at the background levels.

There was no interference between the two loaded beads. As shown in Figure 5, a mixture of the two beads (IgG-PO and IgG-ALP loaded beads) did not result in any false positives when the mixture was tested

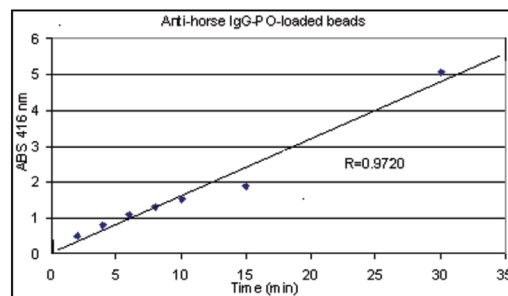
for IgG-PO using the OPD solution and reading the absorbance at 416 nm, or tested for IgG-ALP using the NBT solution and reading the absorbance at 614 nm. This indicates that these two beads can be used in a multiple assay system.

The rate of reaction between IgG-PO and the OPD solution, or IgG-ALP and the NBT solution, under the condition that the two conjugated antibodies were loaded onto the beads, was determined for 30 min. As shown in Figures 6 and 7, the correlation coefficient for IgG-PO is 0.9720 and for IgG-ALP is 0.9717. These values are almost identical, for the same time period, to those of two conjugated antibodies in solution (0.9783 and 0.9768 for IgG-PO and IgG-ALP, respectively). This indicates that the enzymatic reactions on individual beads occur at the same rate as those in solution.

## Conclusion

In this study, the authors have shown that Cavilink polymer beads can be used successfully in a diagnostic assay system. Beads were loaded with either anti-horse IgG-PO or anti-horse IgG-ALP. The maximum loading level for anti-horse IgG-PO was 19.48 µg/g bead and for anti-horse IgG-ALP was 12.01 µg/g bead. The sensitivity of the detection was 4.46 pg/bead for anti-horse IgG-PO and 1.74 pg/bead for anti-horse IgG-ALP.

The reaction time required was 2 min or less. It was noted that the enzymatic



**Figure 6** Reaction rate for the PO-urea-H<sub>2</sub>O<sub>2</sub>-OPD system on the anti-horse IgG-PO loaded beads. Beads loaded with anti-horse IgG-PO were reacted with the urea-H<sub>2</sub>O<sub>2</sub>-OPD substrate solution and results were monitored for 30 min.

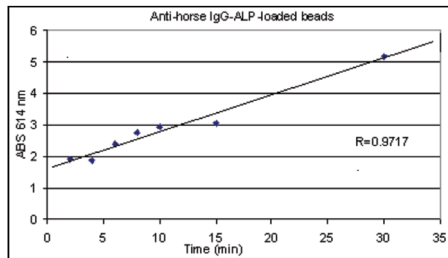


Figure 7 Reaction rate for the ALP-BCIP-NBT system on the anti-horse IgG-ALP loaded beads. Beads loaded with anti-horse IgG-ALP were reacted with BCIP-NBT substrate solution and results were monitored for 30 min.

reaction rate on individual beads is similar to the rate found in solution. Also, since reactions occur on individual beads, they can be used either in a nanoscale multiple assay system without cross-bead interference, or can be embedded into strips for strip assay systems.

Based on this preliminary work, it appears that the structural features of Cavilink porous polymers make them useful as a matrix for binding and reaction with various macromolecules. The authors suspect that the very large, micrometer-size cavities allow biopolymers to maintain their native configuration and therefore enhance efficiency of reaction with epitopes.

## References

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