

Improved Detection Sensitivity in ELISAs by Enzyme Multi-labeled DNA Dendrimers Conjugated to Anti-biotin Antibodies

Johanna R. Mora and Robert C. Getts
Genisphere Inc. 2801 Sterling Dr, Hatfield, PA 19440

Abstract

Enzyme-linked immunosorbent assays (ELISAs) are simple and inexpensive methods used to determine the presence of biomarkers for the diagnosis of multiple diseases and drug development. Output signal depends on the number of enzyme molecules that can be indirectly attached per analyte molecule. Improving the detection sensitivity of the assay would allow a better understanding of cellular mechanisms and decrease the amount of sample required per assay. To increase the assay's sensitivity other groups have tried successive incubations of enzyme labeled avidin and biotinylated anti-avidin antibodies. The main drawbacks of this approach are an increase in assay time and decrease in reproducibility due to the multiple incubations and washes. A better approach would be to deliver a higher number of labels. To this end, we have prepared DNA dendrimers conjugated to anti-biotin antibodies containing up to 300 molecules of horseradish peroxidase (HRP), and compared these reagents to streptavidin-HRP (~2HRP/streptavidin). The assay format consisted of a standard microtiterplate ELISA in which the dendrimer conjugate is a "drop-in" reagent replacing the streptavidin, so that no extra steps are involved. Interleukin 1 alpha (IL-1a) was selected as the model analyte. Upon optimization we were able to detect less than 2 pg/mL of IL-1a, corresponding to greater than 20-fold signal amplification respect to streptavidin-HRP. The conjugate was also used to detect IL-6, TNF alpha, antibodies against beta 2 glycoprotein 1 (B2 GPI) and gastric parietal cell antibody (GPA). For these markers signal amplifications ranged from 3 to 218 fold compared to streptavidin-HRP detection.

Keywords: DNA dendrimers, ELISA, cytokines, B2 GPI antibodies, GPA antibodies, signal amplification

Background

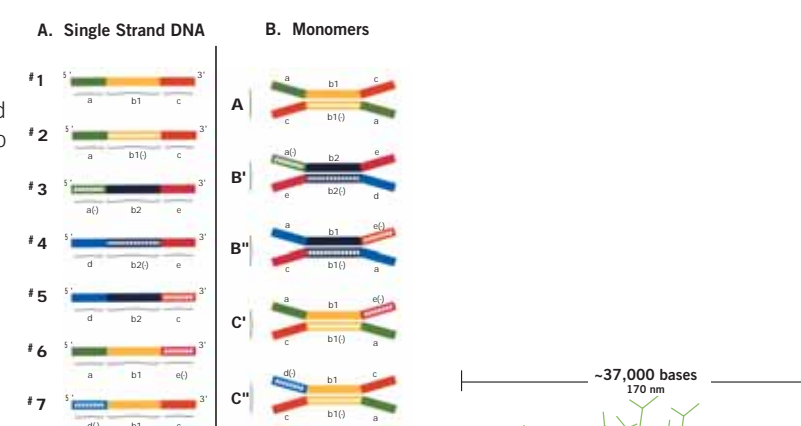
Importance of Signal Amplification

Lower limits of detection and greater sensitivity translate into a better understanding of biological systems. O'Connor and co-workers¹ increased the sensitivity of detection by successive incubations of enzyme labeled avidin and biotinylated anti-avidin antibodies. This results in longer assays and lower reproducibility due to the multiple incubations and washes.

Our approach consists in increasing the number of enzymes per detection antibody by using DNA dendrimers² conjugated to anti-biotin antibodies, and labeling them with up to 300 enzymes (horseradish peroxidase).

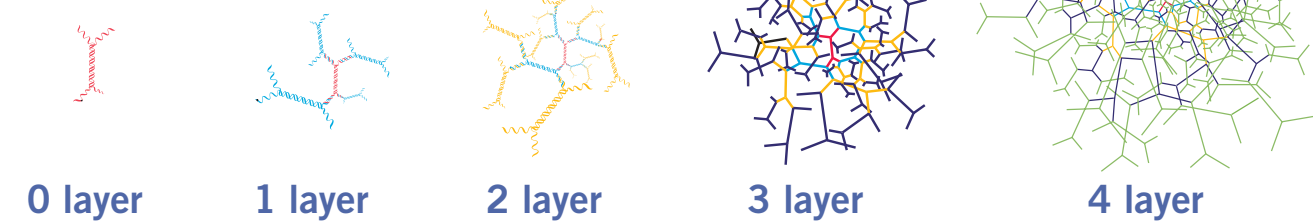
Dendrimer Components

Dendrimers are made from 7 single stranded DNAs. The strands are hybridized pair-wise to produce building block units called monomers.



Dendrimer Technology

Monomers are hybridized together to assemble various size DNA dendrimers. The "core" structure is crosslinked during assembly to form a completely covalent structure.

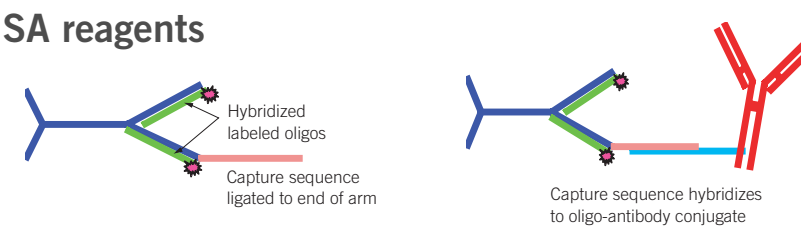


Methods

Preparation of dendrimer ELISA reagents

Enzyme and Antibody labeling

The dendrimers were incubated at room temperature for 30 minutes in SuperFreeze™ (Pierce, IL) buffer with HRP and anti-biotin antibodies labeled oligonucleotides complementary to the outer core of the dendrimer.



ELISA Assays

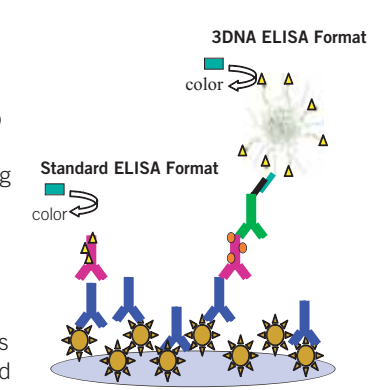
Indirect ELISA

Antigen-coated plates from INOVA were used to test indirect ELISAs. The assay was done according to the manufacturer.

For dendrimer approach, the incubation with the HRP labeled anti-human antibody was substituted by biotinylated anti-human antibody.

Unbound antibodies were washed off and samples were incubated with the anti-biotin dendrimer conjugate (30ng as dendrimer) for 1 hour.

The wells were washed and incubated with substrate. The reaction was stopped after 20 minutes and the absorbance read at 450 nm.

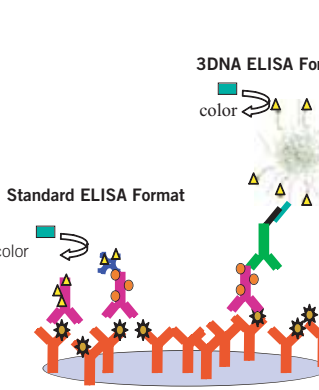


Sandwich ELISA

Pre-coated plates from Pierce were used to test the sandwich approach. All assays were run according to the manufacturer.

For dendrimer detection, streptavidin-HRP was substituted by incubation with anti-biotin HRP-labeled dendrimer (18 ng as dendrimer) for 1 hour at room temperature.

The wells were washed and incubated with substrate. The reaction was stopped after 20 minutes and the absorbance read at 450 nm.



Results

Figure 1

Optimization of anti-biotin Dendrimer conjugate preparation.

(a) Anti-biotin oligonucleotide titration. The HRP oligonucleotide to dendrimer molar ratio was kept constant.

(b) HRP oligonucleotide titration. The dendrimer was incubated with two types of HRP oligonucleotides. The anti-biotin oligonucleotide to dendrimer ratio was fixed for all samples.

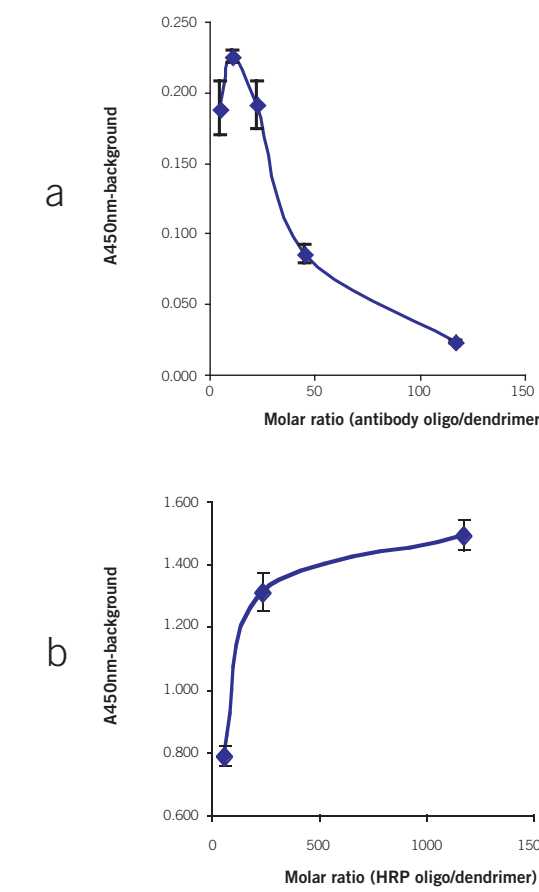


Figure 2

Effect of NaCl in Guardian Peroxidase as the dendrimer binding buffer. Dendrimer conjugates were prepared at 6.6 ng/μL and diluted to 0.6 ng/μL in buffer prior to incubation with biotin-target. The conjugates were tested for detection of 64 pg/mL of IL-1a.

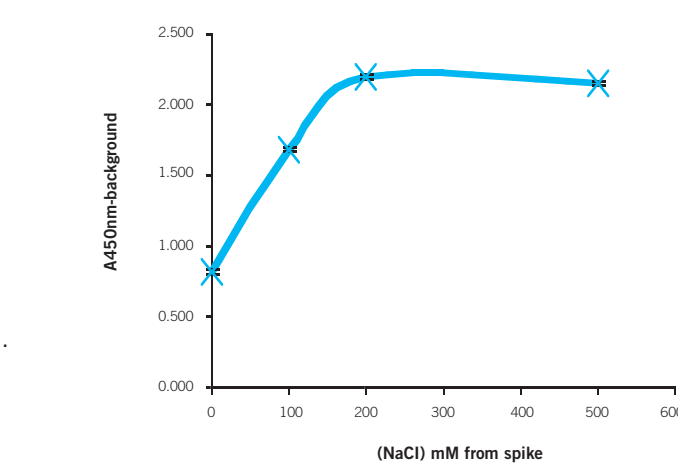


Figure 3

Evaluation of dextran sulfate in the dendrimer binding buffer. A 4-layer dendrimer conjugate was diluted in binding buffer, and each well was incubated with 50 μL of the hybridization mixture.

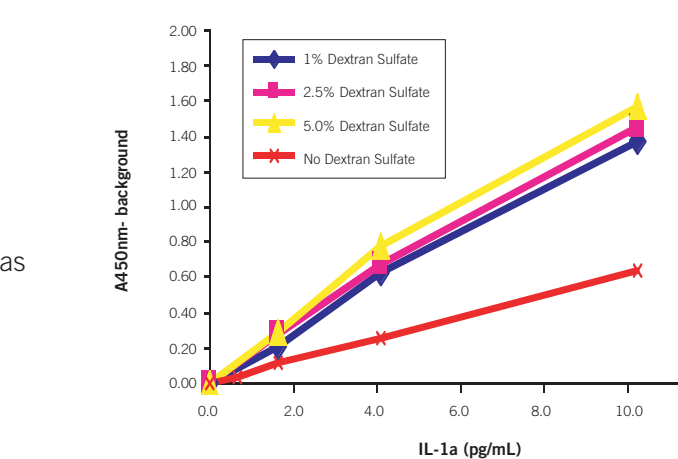


Figure 4

Effect of dendrimer conjugate mass on signal. The dendrimer conjugate was diluted in hybridization buffer, and each well was incubated with 50 μL of the hybridization mixture.

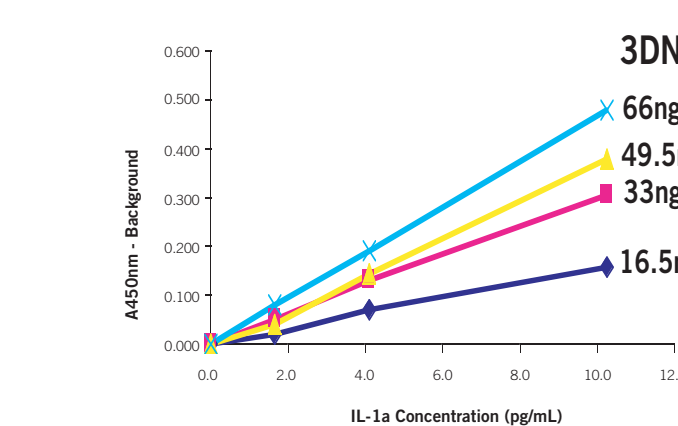


Figure 5

Evaluation of time and agitation during dendrimer conjugate incubation. The conjugates were tested for IL-1a detection. The conjugate mass was fixed at 18 ng as dendrimer per well.

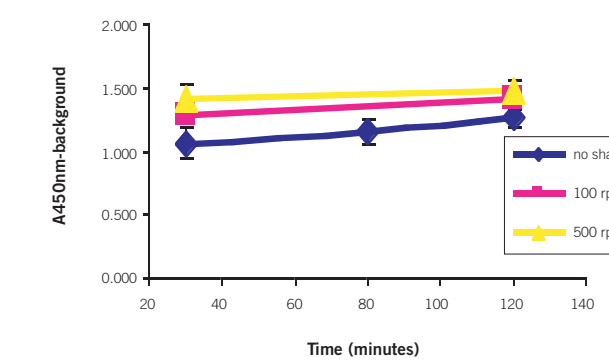


Figure 6

Stability study of a HRP-labeled anti-biotin dendrimer. The conjugate was stored in 50mM Tris, 150 mM NaCl at 310, 298 and 277 Kelvin. Zero order kinetics were assumed to calculate the half life at each temperature.

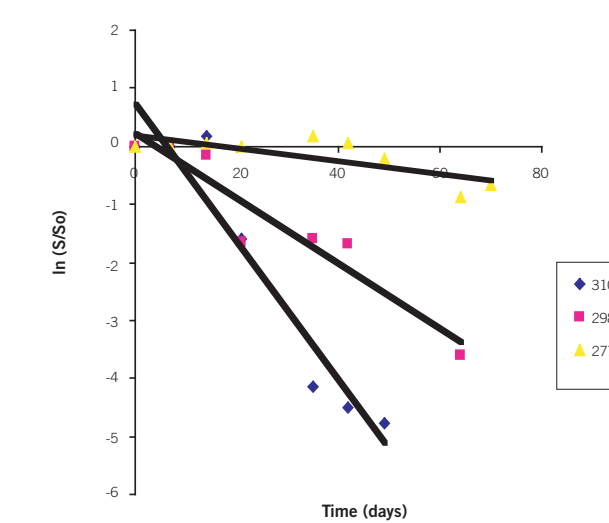


Figure 7

Detection of gastric parietal cell (H+/K+) ATPase antibodies by 2-layer and 4-layer dendrimers compared to the standard assay. (a) Evaluation of 2-layer and 4-layer anti-biotin dendrimer conjugates. (b) Same assay comparing the response from an anti-biotin dendrimer conjugate to that of an anti-human IgG dendrimer conjugate.

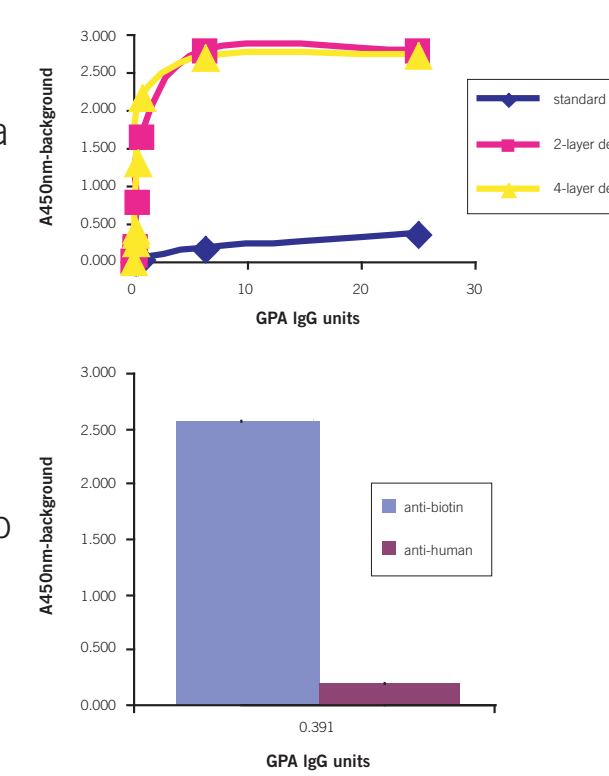
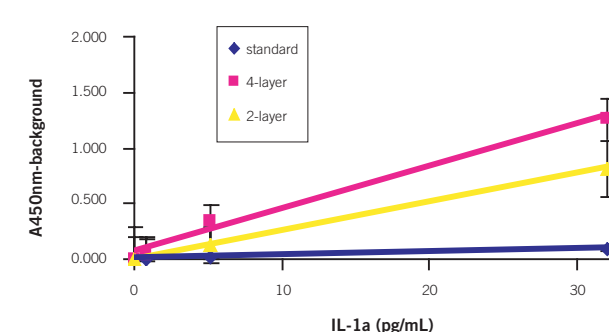


Figure 8

Comparison of signal response from 2-layer and 4-layer anti-biotin dendrimer conjugate compared to signal from a standard Pierce assay for IL-1a detection. 18 ng of conjugate as dendrimer were used for the detection in a 50 μL volume.



Results

Table I.

Summary of signal amplification obtained with dendrimer conjugate detection with respect to standard methods.

ELISA	Kit/Ab pair	Highest amplification	Correlation (R ²)
Indirect	H pylori	10	0.999
Indirect	Histone	7	0.995
Indirect	GPA	218	0.985
Indirect	ACA	17	0.999
Indirect	B2GPI	30	0.996
Sandwich	IL-1a	80	0.997
Sandwich	IL-6	15	0.996
Sandwich	TNF	3	0.999

Summary

- The optimal HRP oligonucleotide to dendrimer molar ratio range is 24 to 39 for 2-layer dendrimers and 235 to 390 for 4-layer dendrimers.
- The optimal anti-biotin oligonucleotide to dendrimer molar ratio is 12 for 2-layer dendrimers and 50 for 4-layer dendrimers. Unbound-excess antibody would compete with dendrimer conjugates for biotin binding.
- NaCl concentration, dextran sulfate percentage, and overall conjugate concentration in the binding buffer were determined to be important parameters in the optimization of dendrimer conjugate binding to target.
- Agitation during dendrimer conjugate incubation increases the output signal presumably by improving the kinetics of dendrimer conjugate binding to target.
- The half life of the dendrimer conjugate prepared in TBS and stored at 253 K and 277 K is approximately 60 days. Preliminary results indicate that the conjugate's half life at 253 K when stored in SuperFreeze™ is over 300 days.
- As expected, 4-layer dendrimer conjugates give greater signal amplification than the 2-layer equivalent.
- Signal amplification varies depending on the antibody pair and possibly the proximity of the target to the plate.

References

- O'Connor, E., Roberts, E. M., and Davies, J. D. Amplification of cytokine-specific ELISAs increases the sensitivity of detection to 5-20 picograms per milliliter. *J. Immunol. Methods* (1999) 229:155-160.
- Nilsen, T.W., Grayzel, J., and Prenskey, W. *Dendritic Nucleic Acid Structures*. *J. Theor. Biol.* (1997) 187: 273-284.
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