A versatile solid support system for oligodeoxynucleotide probe-based hybridization assays

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ABSTRACT

A procedure for immobilization of well-defined quantities of oligodeoxyribonucleotides (ODNs) to a versatile nylon support is described. The solid support, a nylon-6/6 bead, is covalently coated with poly(ethyleneimine) to provide a reactive spacer-arm for attachment of ODNs. 5'-Aminohexyl-tailed ODNs are selectively activated using 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride) and then covalently attached to the bead via the triazine moiety. The modified nylon support has a low level of binding of nonspecific nucleic acid and efficiently captures both RNA and DNA targets.

INTRODUCTION

Nucleic acid hybridization provides exquisite specificity for the recognition of DNA and RNA sequences and has become a powerful technique in the diagnosis of infectious diseases. With the advent of automated synthesis of short, synthetic oligodeoxynucleotide (ODN) probes;[1] it is now possible to develop rapid, simple, and inexpensive diagnostic assays based on nucleic acid hybridization. The use of DNA probes in analytical techniques has been reviewed.[2]

The most commonly used methods for the detection of specific nucleic acid sequences typically involve the immobilization of the target nucleic acid on solid supports, such as nitrocellulose filters or nylon membranes, followed by hybridization with an appropriately labeled signal probe. The disadvantages of this method are that the immobilized nucleic acid is not covalently attached which can result in loss of the nucleic acid from the support. Also, only a small amount of the nucleic acid is available for hybridization. These problems can be overcome by using a 'sandwich' hybridization assay. In a sandwich-type assay[3,4] the 'target' nucleic acid (analyte) is hybridized to a 'capture' ODN probe that has been covalently immobilized on a solid support. A second 'signal' nucleic acid probe, typically labeled with a radioisotope or a reporter group, is hybridized to a different region of the captured analyte nucleic acid.

A significant barrier to the more widespread use of DNA probes in 'user friendly' assays has been the lack of solid supports and immobilization methods that are fully compatible with the hybridization process. The use of solid supports in DNA probe-based hybridization has been reviewed.[5] Several methods for covalent attachment of oligonucleotides to solid supports have been developed.[6-8]

We report here an efficient and highly reproducible method for the immobilization of capture ODNs on poly(ethyleneimine)-modified nylon beads using cyanuric chloride as the coupling reagent. This procedure allows for the efficient and reliable modification of 5'-amine-tailed ODNs selectively on the terminal amine. After modification with cyanuric chloride the ODNs can be readily immobilized on an amine containing solid support. The cyanuric chloride modified ODNs can be stored for up to one week at 4°C in a buffered aqueous solution (pH 8.3) with little decomposition.

The poly(ethyleneimine) (PEI) coating imparts the necessary secondary and tertiary structure to the bead surface to permit immobilization of high concentrations of ODNs and efficient capture of target nucleic acid from solution. The derivatized nylon bead supports have significant advantages over most of the solid supports commonly used to immobilize capture nucleic acid sequences. The ODN-modified beads exhibit: (1) excellent hybridization kinetics, (2) very low protein and nonspecific nucleic acid affinity, (3) high ODN capacity, (4) very low production cost, and (5) are stable to all commonly used hybridization formats (including heating to 90°C for 15 min.). The use of the nylon bead support for DNA probe assays allows for great versatility in the design and handling of the assay format.

MATERIALS AND METHODS

Sodium borate buffers (SBB) were freshly prepared from boric acid and sodium hydroxide. APB buffer is 0.18 M NaCl, 0.05 M Tris pH 7.6, 5 mM EDTA, and 0.5% Tween 20® . TMNZ buffer is 0.05 M Tris pH 9.5, 1 mM $MgCl_2$, 0.5 mM $ZnCl_2$. FW (filter wash) is 0.09 M NaCl, 50 mM Tris pH 7.6, 25 mM EDTA. SDS/FW is FW with 0.1% sodium dodecyl sulfate (SDS). Lysis and hybridization solution is 3 M guanidinium thiocyanate, 2% N-lauroylsarcosine (sarcosyl), 50 mM Tris pH 7.6, and 25 mM EDTA. CAP buffer is 0.1 M sodium citrate and 0.2 M sodium phosphate, pH 6.5. HRP (horseradish peroxidase) substrate solution is 0.1 M sodium citrate pH 6.5,

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0.2 M sodium phosphate, 2.87 mM 4-methoxy-1-naphthol,[9] 0.093 mM 3-methyl-2-benzothiazolinone hydrazone hydrochloride[10] and 4 mM hydrogen peroxide. AP (alkaline phosphatase) substrate solution is 1 mM 5-bromo-4-chloroindoyl-3-phosphate, 1 mM nitroblue tetrazolium,[11] and 0.01% Tween 20 in TMNZ. The fluorescent substrate for alkaline phosphatase is 0.5 mM 4-methylumbelliferone phosphate,[12] 0.05 M Tris pH 9.5, 1 mM $MgCl_2$, 0.5 mM $ZnCl_2$. A chemiluminescent cocktail for alkaline phosphatase was a generous gift from Paul Schaap and Lumigen, Inc. (Detroit, MI). Poly(ethyleneimine) was purchased from Polysciences (Warrington, PA). Burnished or unpolished nylon beads were purchased from The Hoover Group (Sault St. Marie, MI). Triethyloxonium tetrafluoroborate, succinic anhydride and 1-methyl-2-pyrrolidinone were purchased from Aldrich Chemical (Milwaukee, WI). Tween 20® and NHS-LC-Biotin were purchased from Pierce (Rockford, IL). Guanidine thiocyanate (GuSCN) was purchased from Kodak (Rochester, NY). Cyanuric chloride was from Aldrich Chemical Co. (Milwaukee, WI) and was recrystallized from toluene.

ODN Synthesis

ODNs complementary to conserved or hypervariable regions[13] of the 16S ribosomal RNA (rRNA) of *Porphyromonas gingivalis* (*Pg*), Table 1, were synthesized on either an ABI 380B or a MilliGen 7500 automated DNA synthesizerKiK using the standard cyanoethyl-N,N-diisopropylamino-phosphoramidite (CED-phosphoramidite) chemistry. Amine tails were incorporated onto the 5'-end using the commercially available *N*-monomethoxytritylaminohex-6-yloxy-CED-phosphoramidite. ODNs were purified by adaptations of standard methods.[1] ODNs with 5'-monomethoxytrityl groups were chromatographed by HPLC using a Hamilton PRP-1 (7.0×305 mm) reversed-phase column employing a gradient of 5% to 45% CH_3CN in 0.1 M $Et_3NH^+OAc^-$, pH 7.5, over 20 min. After detritylation with 80% acetic acid, the ODNs were precipitated by addition of 3M sodium acetate and 1-butanol. Analytical checks for the quality of the ODNs were done by ion-exchange HPLC using a Toso-Haas DEAE-NPR column and by denaturing polyacrylamide gel electrophoresis (PAGE).

Preparation of the Polymer-coated Nylon Bead

Unpolished nylon beads (25,000, 3/32 inch diameter) in anhydrous 1-methyl-2-pyrrolidinone (1800 mL) were stirred for 5 min. at ambient temperature. Triethyloxonium tetrafluoroborate (200 mL, 1 M in dichloromethane) was added and then stirred for 30 min. at ambient temperature. The liquid was decanted and the beads were washed quickly with 1-methyl-2-pyrrolidinone (4×500 mL). The beads were then stirred for 12−24 h in a 3% (w/v) solution (1 L) of 70,000 MW poly(ethyleneimine) in 1-methyl-2-pyrrolidinone (prepared from a 30% aqueous solution of poly(ethyleneimine)). After decanting the poly(ethyleneimine) solution, the beads were washed with 1-methyl-2-pyrrolidinone (2×1 L), SDS/FW (2×1 L), H_2O (10×2 L), and finally with 95% ethanol (1×500 mL). The beads were dried under high vacuum for 4 to 5 h. The amine content of the beads was determined by reaction with picrylsulfonic acid.[14]

Table 1. List of ODN sequences

Name	Sequence	Function
Pg006	5'-XCCTTAGGACAGTCTTCCTTCACGC-3'	*Pg* specific capture probe
UP041	5'-XCTGCTGCCTCCCGTAGGAGT-3'	Universal signal probe
UP007	5'-XGTATTACCGCGGCTGCTG-3'	" "

'X' indicates a 5'-aminohexyl tail

Preparation of 5'-[6-(4,6-Dichloro-1,3,5-triazin-2-ylamino)-hexyl]-ODNs

To a solution of 5'-aminohexyl ODN (1 mL, 10 mg/mL) in freshly prepared 0.1 M SBB (pH 8.3, 3.2 mL) and H_2O (1.8 mL) was added an acetonitrile solution of recrystallized cyanuric chloride (1 mL, 50 mg/mL). The solution was mixed for 30−120 min. at ambient temperature. The unreacted cyanuric chloride was removed by ultrafiltration through a 3000 MW cutoff membrane (Amicon, Beverly, MA) using freshly prepared 0.1 M SBB (pH 8.3, 4×10 mL) as the wash solution. After the final wash the volume was reduced to 1 mL. The 5'-[6-(4,6-dichloro-1,3,5-triazin-2-ylamino)hexyl]-ODNs are stable for 1 week at 4°C in 0.1 M SBB (pH 8.3) with no detectable decomposition.

Attachment of ODNs to Nylon Beads

PEI-coated nylon beads (500 beads), described above, were placed in an equal volume of freshly prepared 0.1 M SBB (pH 8.3) and vigorously agitated for 30 min. to rehydrate the beads. The borate solution was decanted and the beads were washed once with 0.1 MSBB (pH 8.3) then covered with an equal volume of fresh 0.1 M SBB. The borate solution of the 5'-[6-(4,6-dichloro-1,3,5-triazin-2-ylamino)hexyl]-ODN (1 mL, 500 μg/mL) was then added to the beads. The mixture was vigorously agitated at ambient temperature for 60 min. The solution was decanted and the beads were then washed with 0.1 M SBB (pH 8.3, 2×500 mL). The beads were treated in three times the volume of the beads with succinic anhydride (10 mg/mL) in 9:1 1-methyl-2-pyrrolidinone: 1.0 M SBB (pH 8.3). The reaction mixture was stirred for 1 h at ambient temperature. The beads were then washed with 1-methyl-2-pyrrolidinone (3×250 mL), dH_2O (2×1 L), SDS/FW (5×250 mL), and then with dH_2O (4×1 L). The beads were stored in 25 mM EDTA.

Biotinylation of Signal ODNs

To a solution of ODN (UP041 or UP007, 1 mg) in freshly prepared 0.2 M SBB (pH 8.3, 250 μL) was added NHS-LC-Biotin (5 mg in 250 μL of 1-methyl-2-pyrrolidinone). The reaction was incubated overnight at ambient temperature. The biotinylated ODN was then purified by size-exclusion chromatography on Sephadex® G-50 to give a nearly quantitative yield with approximately 95% purity (the remainder is unbiotinylated ODN).

The Hybridization-based Sandwich Assay

Bacterial Lysate Preparation. A 5-fold serial dilution of *Pg* was prepared as follows: A pellet consisting of 1×10^8 cells each of *Actinobacillus actinomycetemcomitans* (*Aa*), *Pg*, *Bacteroides intermedius* (*Bi*), *Eikenella corrodens* (*Ek*), *Fusobacterium nucleatum* (*Fn*), or *Wolinella recta* (*Wr*) was lysed in 200 μL of 3 M GnSCN lysis solution at 19°C. The lysate was then heated to 65°C for 5 min. The lysate was diluted from 1×10^8 to 6.4×10^3 in 5-fold increments using a diluent containing biotinylated signal ODNs (UP041 and UP007) at 1 μg/mL and a mixture of 1×10^8 lysed cells (200 μL) each of *Aa*, *Bi*, *Ek*, *Fn*, and *Wr*. The zero-lysate control consisted of the diluent.

Hybridization Assay with Colorimetric Detection. Nylon beads each covalently immobilized with 0.1 μg of Pg006 specific oligonucleotide probe were incubated in the lysate solutions (3 beads/200 μL lysate) for 30 min. at ambient temperature with vigorous agitation. After decanting the lysates, the beads were then washed once each with lysis/hybridization solution, FW, and SDS/FW. Streptavidin/HRP (a 1:1 covalent conjugate prepared at MicroProbe) conjugate was added to a final concentration of 1 μg/mL (based on streptavidin) in SDS/FW and incubated 10 to 15 min. at ambient temperature with mild agitation. The beads were washed three times with SDS/FW and then once with CAP buffer. The HRP substrate solution, 4-methoxy-1-naphthol, was added and the reaction was allowed to proceed for 15 min. at ambient temperature. The beads were washed once with SDS/FW and then once with FW and allowed to air dry in the dark.

Hybridization Assay with Fluorescence Detection. Nylon beads each covalently immobilized with 0.1 μg of Pg006 specific ODN probe were incubated in the lysate solutions (2 beads/200 μL lysate) for 30 min. at ambient temperature with vigorous agitation. The beads were washed with SDS/FW at ambient temperature, washed with APB, and then incubated with 0.4 μg/mL of streptavidin/alkaline phosphatase (SA/AP) conjugate in APB for 5 min. at ambient temperature. The beads were then washed 5 times with APB, and once with TMNZ. The presence of alkaline phosphatase was determined by incubating the nylon beads with 150 μL of 0.5 mM 4-methyl-umbelliferyl phosphate for 30 min. at 37°C. The solutions were transferred to black microtiter 8-well strips (Dynatek Laboratories, Chantilly, VA) and then read using a Fluoroskan II fluorometer (Flow Laboratories, McLean, VA) using an excitation wavelength of 360 nm and an emission wavelength of 456 nm.

Hybridization Assay with Chemiluminescence Detection. Nylon beads covalently immobilized with 0.1 μg of Pg006 specific ODN probe were incubated in the lysate solutions (3 beads/200 μL lysate) for 30 min. at ambient temperature with vigorous agitation. The beads were washed once with SDS/FW followed by 3 washes with APB. The beads were then incubated with 0.4 μg/mL of streptavidin/alkaline phosphatase (SA/AP) conjugate in APB for 5 min. at ambient temperature. After washing the beads 5 times each with APB and once with TMNZ, the presence of alkaline phosphatase was determined by incubating the beads with 200 μL of chemiluminescent Lumigen substrate (Lumigen, Inc., Detroit, MI) in 5mm×40 mm polypropylene tubes. The signal was detected, in the presence of the bead, using a Turner TD 20e luminometer (Turner Designs, Sunnyvale, CA) with an

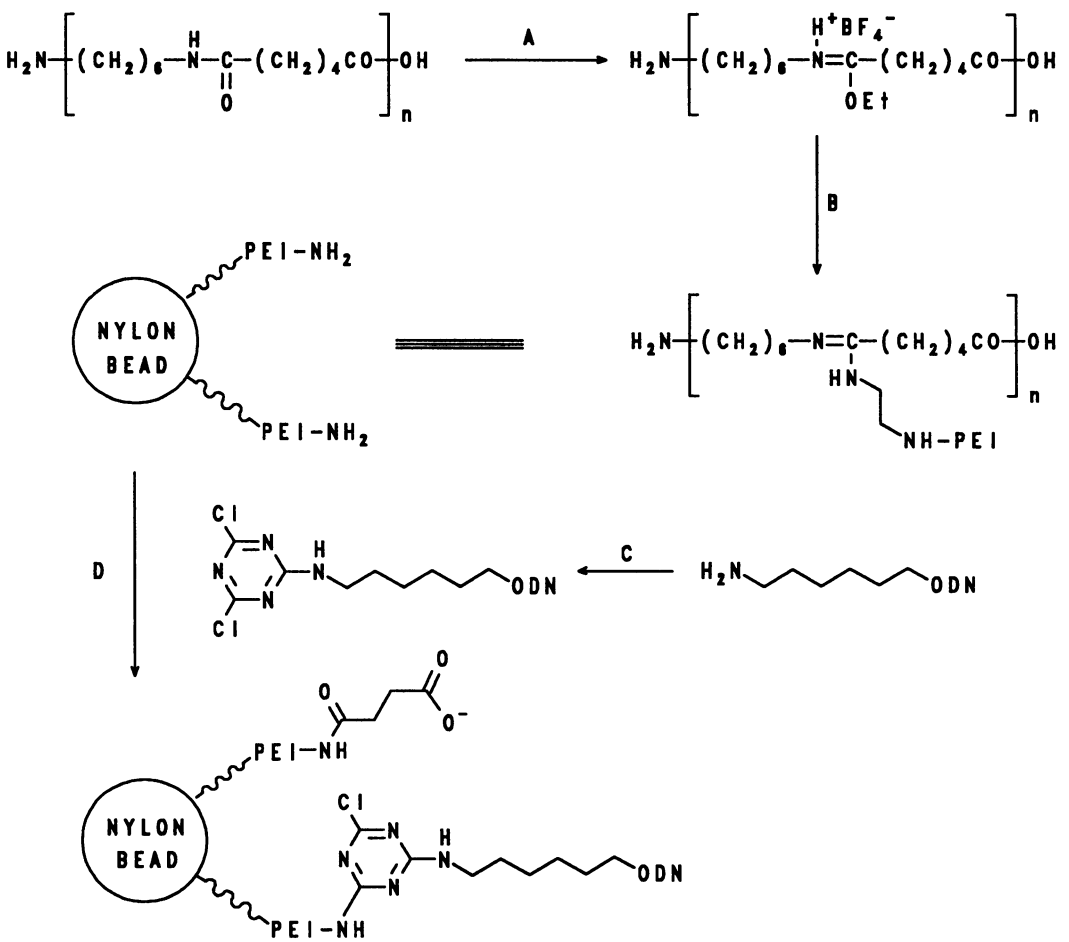

Figure 1. Preparation of ODN-modified Nylon Bead. Step A is the alkylation of the nylon with triethyloxonium tetrafluoroborate. Step B is the coating of the surface with poly(ethyleneimine). Step C shows the activation of the ODN with cyanuric chloride and Step D is the covalent immobilization of the ODN sequence on the bead surface and the subsequent capping of unreacted amines with succinic anhydride.

integration time of 1 min. (The Dynatek chemiluminometer can also be used. Dynatek Laboratories, Chantilly, VA) All values presented in the tables are averages from three to six beads per titration point.

RESULTS AND DISCUSSION

Nylon supports have been used for the covalent attachment of proteins and enzymes[15-21] as well as for the non-covalent immobilization of nucleic acid.[22] We now describe a novel procedure for the covalent attachment of ODNs to a specially modified solid support that provides excellent coupling efficiencies and hybridization properties. The details of each step are discussed below.

Activation of the Nylon Surface Via Alkylation

Our procedure for the synthesis of ODN derivatized nylon beads is shown in Figure 1. The nylon bead is ethylated using triethyloxonium tetrafluoroborate to form amine reactive imidate esters on the surface of the nylon (Figure 1, step A). We have found that as a reaction solvent, 1-methyl-2-pyrrolidinone is preferred to less polar solvents, such as dichloromethane that were used by Carrico et al.[22] to activate nylon beads. Less polar solvents in the presence of the alkylating agent partially dissolve the surface of the bead. Dissolution of the bead surface severely impacts the ODN immobilization and hybridization properties. The bead surface should be unpolished to effect the greatest possible surface area.

The activated bead is then reacted with poly(ethyleneimine) (M_r ~ 10K−70K) to form a polymer coating that provides an extended amine surface for the attachment of ODNs (Figure 1, step B). We found that coating the nylon surface with poly(ethyleneimine) enhances both the efficiency of ODN derivatization and the hybridization of target polynucleotides.

Selective Activation of the 5′-Amine of ODNs with Cyanuric Chloride

Procedures for immobilization of ODNs have been recently reviewed.[23] Methods for immobilization of long strands of nucleic acid have typically involved reaction of the nucleobases with the solid support via diazotization, cyanogen bromide activation, or water-soluble carbodiimide. Recently, cyanuric chloride was used to immobilize genomic DNA to cellulose, and as in the previous methods, the site of attachment to the solid support was the nucleobases.[24,25] Cyanuric chloride has been used for the covalent binding of protein to cellulose[26,27] to immobilize protein and enzymes to Sepharose,[28] and for protein conjugation and immobilization.[29]

We report here that amine-tailed ODNs selectively react with excess cyanuric chloride, exclusively on the amine tail, to give a 4,6-dichloro-1,3,5-triazinyl-ODN in quantitative yield (Figure 1, step C). The displacement of one chlorine moiety of cyanuric chloride by the amino group significantly diminishes the reactivity of the remaining chlorine groups. This results in increased hydrolytic stability of the 4,6-dichloro-1,3,5-triazinyl-ODN. The 4,6-dichloro-1,3,5-triazinyl-ODNs are stable for extended periods in buffered aqueous solutions (pH 8.3, 4°C, 1 week) and are readily isolated and purified by size exclusion chromatography or ultrafiltration.

To ascertain that the nucleobases in the ODN did not react with cyanuric chloride under the reaction conditions, the ODN sequence UP041 was synthesized with and without a 5′-aminohexyl tail and then reacted with cyanuric chloride. The reaction was followed by reversed-phase HPLC and we observed that only the UP041 ODN possessing the 5′-tethered amine reacted with cyanuric chloride. This demonstrates that the reaction is specific for the amine tail with no apparent reaction on the nucleotide moieties (data not shown). The reaction of primary amines with the 4,6-dichloro-1,3,5-triazinyl-ODNs is slow, however the reaction with the PEI-coated bead is very rapid. The improved rate of reaction is probably due to an electrostatic attraction of the anionic ODN for the cationic bead surface that allows the 4,6-dichloro-1,3,5-triazinyl-ODN to come into close proximity to the reactive amines.

ODN-Bead Conjugation

The PEI-coated bead is then reacted with the cyanuric chloride activated ODN. High concentrations of the 'capture' sequence are readily immobilized on the surface of the bead (Figure 1, step D) and the unreacted amines are capped with succinic anhydride in the final step of the derivatization process. It is important to block the unreacted amines since the unblocked polymer-derivatized bead has a significant affinity for nucleic acid. This is due to the high surface concentration of 1°, 2°, and 3° amines that imparts a net positive charge to the surface. We have found that succinic anhydride is the preferred capping reagent since it imparts a net neutral (zwitterionic) to negative charge to the bead surface. The resulting solid support has almost no measurable non-specific binding of nucleic acid.

The quantity of immobilized oligonucleotide was determined by spiking the capture ODN with a small amount of ^{32}P-labeled capture (3′ labeled using terminal transferase)[30] oligonucleotide prior to activation with cyanuric chloride. After determining the specific activity of the oligonucleotide and then measuring the amount of radioactivity bound per bead, the quantity of

Table 2. Quantity of covalently immobilized ODN as a function of input mass of 4,6-dichloro-1,3,5-triazinyl-ODNs

ODN Mass			
Input (μg)	Immobilized (μg)	% Immobilized	LLD
10.0	1.2	11%	8×10^5
2.0	0.8	39%	8×10^5
1.0	0.5	47%	8×10^5
0.2	0.12	62%	8×10^5
0.1	0.06	62%	8×10^5

Mass values are on a per 3/32nd inch bead basis. LLD indicates the lowest level of detection of *Pg* bacterial cells in HRP-based sandwich assay.

Table 3. Specific detection of *P. gingivalis* 16S rRNA using colorimetric, chemiluminescent, or fluorescent signal systems

		Relative Intensity Units	
Cell number	Colorimetric (HRP)	Chemiluminescent	Fluorescent
1.0×10^8	+++	off scale	1870
2.0×10^7	+++	off scale	1140
4.0×10^6	++	1650	630
8.0×10^5	+*	680	160
1.6×10^5	−	320	60
3.2×10^4	−	260*	27*
6.4×10^3	−	210	24
control	−	200	17

* indicates the lower limit of detection (defined as control + 3×SD).

immobilized ODN was measured. The quantity of covalently immobilized ODN is proportional to the quantity of activated ODN reacted with the derivatized nylon support. The quantity of ODN bound as a function of ODN concentration in the immobilization procedure is shown in Table 2.

We have used other polyamines (i.e., hexanediamine, lysine, pentaethylenehexamine, poly(allylamine), poly(vinylamine), etc.) in place of PEI. These amines provide excellent ODN loading levels and can, like the PEI derivatized beads, be stoichiometrically titrated with complementary ODN sequence, indicating full accessibility of the bound sequences for hybridization to oligonucleotides. However, the PEI-coated beads were approximately 25-fold more efficient in the capture of 16S rRNA compared to other polyamine derivatized beads (data not shown). This suggests that the tertiary structure of PEI imparts some property to the bead surface that enhances capture of larger, target nucleic acids. Therefore, the ability to capture a complementary short ODN may not reflect the ability to efficiently hybridize to or capture long denatured DNA or rRNA.

Hybridization Assay Performance of the Nylon Beads

The performance of the bead was determined by comparing the quantity of rRNA hybridized as a function of ODN loading (Table 2). ODN loading levels as low as 0.05 μg/bead retain the ability to efficiently hybridize 16S rRNA as demonstrated by determining the lowest levels of detection (LLD) of target *Pg* rRNA. We have found that 0.01 to 0.05 μg/bead is the lowest level of immobilized ODN that will efficiently hybridize complementary polynucleotides over a wide range of target polynucleotide concentrations (10^6-10^{13} targets).

Compatibility of the Nylon Solid Support with Colorimetric, Chemiluminescent, and Fluorescent Signal Systems

The results from the specific hybridization and detection of 16S rRNA in crude GuSCN lysates in a sandwich assay format are described in Table 3. Beads containing 0.2 μg/bead of covalently immobilized *Porphyromonas gingivalis* (*Pg*) capture ODN,[31] were incubated in a lysate containing five types of non-homologous bacterial species. The results described in Table 3 indicate that 8×10^5 *Pg* cells were detectable using HRP with colorimetric detection. The total assay time was less than 1 h. No non-specific background was observed after incubation with the crude lysate containing large numbers of non-specific bacterial species. When using alkaline phosphatase, 5-bromo-6-chloro-indoyl phosphate, and nitroblue tetrazolium as the signal system in the sandwich assay; the LLD was improved approximately 10-fold in a 6 hour assay (5.5 h substrate incubation). Non-specific background was not observed on the control beads, and it has been our general experience that streptavidin conjugates of either HRP or alkaline phosphatase (commercially available or in-house preparations) have very low non-specific affinity for the modified nylon solid supports.

The results of a sandwich assay using ODN-derivatized nylon beads and a chemiluminescent signal detection system are shown in Table 3. The newly developed chemiluminescent substrate 4-methoxy-4-(3-phosphoryloxyphenyl)spiro[1,2-dioxetane-3,2′-adamantane] (PPD)[32] was employed. Using the identical hybridization conditions as described for the colorimetric assay, the nylon beads specifically detect target analyte with a sensitivity exceeding 10,000 cells, or a target number of approximately $1-5\times10^7$.

We have observed that the dephosphorylated dioxetane (4-methoxy-4-(3-phosphoryloxyphenyl)spiro[1,2-dioxetane-3, 2′-adamantane]) product has no affinity for the modified nylon bead. The dioxetane product freely diffuses into solution, allowing thesignal to be directly detected in the presence of the bead using a single tube chemiluminometer or a 96-well microtiter plate chemiluminometer (Dynatek, Chantilly, MA).

The nylon beads are also compatible with a fluorescent-based signal system. Table 3 also describes target rRNA detection in a sandwich assay using fluorescence detection. The assay format is identical with that described above for the chemiluminescent signal system, except replacing the dioxetane substrate with 4-methyl-umbelliferone phosphate. In a 30 min. hybridization and a 30 min. substrate incubation, 3×10^4 cells were detected when measuring the fluorescence in the absence of the bead. An additional 10-fold increase in sensitivity can be obtained by extending (5−6 h) the substrate incubation times.

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