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Optimization of DNA-tagged liposomes for use in microtiter plate analyses

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Abstract Dye-encapsulating unilamellar DNA oligonucle-otide-tagged liposomes were prepared and characterized for use as signal-enhancing reagents in a microtiter plate sandwich-hybridization analyses of single-stranded RNA or DNA sequences. The liposomes were synthesized using the reversed-phase evaporation method and tagged with DNA oligonucleotides by adding cholesteryl-modified DNA reporter probes to the initial lipid mixture. Liposomes were prepared using probe coverages of 0.0013–0.103 mol% of the total lipid input, several hydrophobic and poly(ethylene glycol)-based spacers between the cholesteryl anchor and the probe, and liposome diameters ranging from 200 nm to 335 nm. Their signal enhancement functionality was compared by using them in microtiter plate sandwich-hybridization assays for the detection of single-stranded DNA sequences. In these assays, an optimal reporter probe concentration of 0.103 mol%, a liposome diameter of 274 nm, and a phospholipid concentration of 0.3 mM were found. The length between the cholesteryl anchor and the probe was optimal when a spacer composed of TEG+$(CH_2O)_3$ was used. Under optimal conditions, a detection limit of 0.5 nM for a truncated synthetic DNA sequence was found with a coefficient of variation of 4.4%. A 500-fold lower limit of detection using fluorescence was found using lysed dye-encapsulating liposomes versus a single fluorescein-labeled probe. Finally, when this method was applied to the detection of *atxA* RNA extracted from *E.coli* SG12036-pIu121 and amplified using NASBA, a minimum extracted concentration of RNA of $1.1×10^{-7}$ µg/µL was found.

Abbreviations

Cholesteryl-TEG	1-dimethoxytrityl-3-*O*-(*N*-cholesteryl-3-amino-propyl)-triethyleneglycolyl-glyceryl-2-*O*-(2-cyanoethyl)-(*N,N*-diisopropyl)-phosphoramidite
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPG	1,2-dipalmitoyl-*sn*-glycero-3[phospho-*rac*-(1-glycerol)],sodium salt
NASBA	nucleic acid sequence-based amplification
SRB	sulforhodamine B
SSC	sodium saline citrate

Introduction

Nucleic acid-based identification is an important tool in the study of infectious diseases, genetic abnormalities, forensics, and biowarfare agent identification [1, 2]. Assays based on sandwich-hybridization are available in several platforms, such as lateral flow assays [3]. Sandwich-hybridization assays enable separation of bound from unbound signaling labels and potentially higher specificity than assays incorporating single detection probes. Mechanistically, these assays rely on the target nucleic acid forming a complex between an immobilized oligonucleotide capture probe and a labeled oligonucleotide reporter

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probe through specific base pairing. Labels for nucleic acid diagnostics ideally yield reproducible, rapid, and sensitive analytical assays and have included radioisotopes, fluorescent, and chemiluminescent markers; enzymes; and nanoparticles such as quantum dots [4, 5]. Each of these labeling methods has proven to provide a suitable means for detection. However, alternatives are warranted given the safety considerations, storage stability, and expense of some of these methods. The advantages of dye-encapsulating liposomes as a label for nucleic acid sandwich-hybridization assays have been elucidated [3, 6, 7]. Such advantages (mainly over enzymatic or radiolabeling approaches) include long-term stability of the encapsulated signaling molecules; substantial and instantaneous signal enhancement through the release of hundreds of thousands of hydrophilic dye molecules from their aqueous cores; and the ease of labeling through the direct incorporation of hydrophobically modified nucleic acid probes into their lipid bilayers.

We reported previously on the development and optimization of a method utilizing cholesteryl-tagged DNA oligonucleotides for tagging dye-encapsulating liposomes and the application of the resulting liposomes in lateral flow assays (LFA) [3]. These optical biosensors utilized liposomes as a signaling mechanism for the detection of RNA from pathogens using sandwich-hybridization and have been applied to the detection of a number of different organisms of environmental and clinical concern [8–11]. Microfluidic devices utilizing liposomal detection based on the sandwich-hybridization principle have also been reported [12, 13]. The LFA biosensor assays rely on the sandwich-hybridization of a nucleic acid sequence-based amplified (NASBA) RNA target between a membrane immobilized capture probe and a visible dye (sulforhodamine B (SRB))-encapsulating liposome conjugated reporter probe. NASBA uses the enzymes avian myeloblastosis virus reverse transcriptase (AMV-RT), RNaseH, and T7 DNA-dependent RNA polymerase in the presence of deoxyribonucleoside triphosphates and appropriate primers to amplify relatively few copies of target RNA into billions of antisense copies isothermally at 41 °C within 90 min [14]. The advantages of NASBA over other amplification techniques such as PCR or RT-PCR include its rapid and selective amplification of RNA, isothermal conditions, and the generation of single-stranded RNA [15, 16].

To our knowledge, liposomes have not been thoroughly investigated as detection reagents for sandwich-hybridization in the high-throughput 96-well microtiter plate format. We, therefore, present here a format in which the capture probe was immobilized to a neutravidin-linked plate through a biotin modification, target DNA or NASBA amplified RNA was then added, followed by the addition of DNA-tagged dye-encapsulating liposomes. The liposomes

were then lysed with surfactant to allow for sensitive, specific, and quantitative measurement of the target molecule (Fig. 1).

In this work, the use and optimization of dye-encapsulating liposomes with respect to size, concentration, degree of probe coverage, and probe spacer length in the sandwich-hybridization microtiter plate format is described. We also demonstrate the advantage of lysed liposomes as a means for detection of single-stranded DNA after release of their encapsulated dye versus intact liposomes and reporter probes with a single fluorophore label. Subsequently, the optimized system was applied to the detection of *Bacillus anthracis atxA* mRNA expressed in an *E. coli* strain and amplified using NASBA in order to prove the potential of this method for lab-based routine sample analysis.

Materials and methods

DPPG, DPPC, DPPE-NBD, and the extrusion membranes were purchased from Avanti Polar Lipids (Alabaster, AL). Streptavidin and sulforhodamine B (SRB) were purchased from Molecular Probes, Inc. (Eugene, OR). All other reagents used in these experiments were purchased from VWR (Bridgeport, NJ). The DNA sequences listed in Table 1 were purchased from Operon Biotechnologies, Inc. (Alameda, CA).

The NASBA enzyme cocktail was purchased from Life Sciences, Inc. (St. Petersburg, FL) and the nucleotides used in the reaction were obtained from Bioline (Randolph, MA) *E. coli* SG12036-pIu121, which expresses the *atxA* gene, was purchased from ATCC (Manassas, VA).
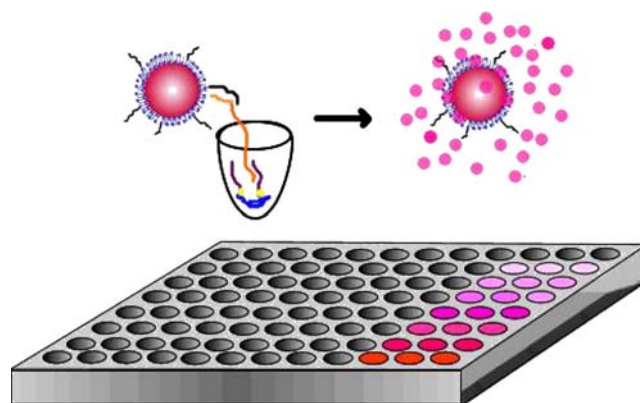

Fig. 1 Schematic of the microtiter plate sandwich-hybridization assay. A biotinylated 22-mer DNA oligonucleotide (*yellow/purple*) was added to neutravidin-coated wells (*blue*). Target RNA or synthetic DNA (*orange*) was then permitted to hybridize. Unbound materials were removed prior to the addition of reporter probe-tagged dye-encapsulating liposomes. The fluorescence at λ_{ex}=540 nm and λ_{em}=590 nm was measured both before and after the addition of 30 mM *n*-octyl-*β*-D-glucopyranoside which was used to lyse the liposomes and release entrapped dye

Table 1 Oligonucleotide sequences

Probe name	Sequence (5′–3′)
Synthetic target	ATAAATACgCggACATCTTgTCTTCTCTTCCCgATATTTCTAg
Cholesteryl-modified reporter probe	CAAgATgTCCgCgTATTTAT-TEG-Cholesteryl
Fluorescein-modified reporter probe	CAAgATgTCCgCgTATTTAT-Fluorescein
Biotinylated capture probe	Biotin-CTAgAAATATCgggAAgAgAA
NASBA primer 1	AATTCTAATACgACTCACTATAgggAgAAggggggAAACggCCAATAATCA
NASBA primer 2	TCAAATTTgCgAAgAACTTgTA

The liposome size distribution was determined by dynamic light scattering using a DynaPro LSR (Proterion Corporation, Piscattaway, NJ) using the Cumulants method of analysis [17, 18]. Diameters reported throughout this report are the average of an assumed monomodal size distribution with its associated size distribution. The phospholipid concentration was determined using Bartlett assays [19]. Optical density measurements were made by diluting 5 μL liposomes with 995 μL of the osmolality-adjusted HEPES buffer (described further below) in an 1.5-mL spectrometer cell at 532 nm. Fluorescence measurements were made using a Bio-Tek FLX800 microtiter plate reader.

Liposome preparation

DPPC, DPPG, and cholesterol (40.3, 21, and 51.7 μmol, respectively) were first dissolved in a 50-mL round-bottom flask in a solvent mixture containing 3 mL chloroform, 0.5 mL methanol, and 3 mL isopropyl ether and sonicated for 1 min at level 6 in a bath sonicator (VWR Aquasonic Model 150D) to ensure homogeneous mixing. Cholesteryl-labeled reporter probe was diluted to a concentration of 300 pmol/μL in a 1:4 (v/v) mixture of methanol/formamide. A 50-μL aliquot of this cholesterol-labeled reporter probe (corresponding to 0.013 mol% of the total lipid input unless otherwise specified) solution was also added to the dissolved lipids. Liposomes incorporating oligonucleotides labeled with a hydrocarbon or a PEG-based spacer between the probe and cholesteryl modification were prepared using the same procedure. A 45 °C solution of dye (2 mL sulforhodamine B, 150 mM in 0.2 M HEPES) was added to the lipid mixture while sonicating for a total of 4 min. The mixture was then placed onto the rotary evaporator and the solvent was removed at 45 °C. The mixture was then transiently vortexed preceding and following an additional introduction of 2 mL 45 °C 150 mM SRB. The mixture was then returned to the rotary evaporator before being extruded at 60 °C 19 times through 2.0-μm membranes, followed by 19 times through 0.6-μm membranes. These initial extrusions were also performed in cases where the liposomes were subsequently extruded through smaller pore size membranes. The liposomes were then passed through a 20×1.7-cm column packed with Sephadex G-50 at ca. 4 mL/min using 1×HEPES-saline-sucrose buffer (10 mM HEPES, 200 mM sodium chloride, 0.01% sodium azide at pH 7.0), osmolality adjusted with sucrose (ca. 0.22 M) to 75 mmol/kg greater than the SRB encapsulant, which was typically about 550 mmol/kg. The liposome fractions containing high liposome densities (usually A_{532}>1.0, OD value obtained using 5 μL liposomes in 995 μL 1×HEPES saline-sucrose) were then combined and dialyzed overnight against the sucrose-HEPES-saline buffer.

Bartlett assays

The phosphorous content for each liposome batch was determined using the Bartlett assay [19]. A sample of liposomes (20 μL) was dehydrated at 180 °C for 10 min, then mixed and heated with 0.5 mL 3.33 N H_2SO_4 for 2 h at the same temperature. A 100-μL aliquot of 30% hydrogen peroxide was added and the mixture was returned to the oven for 1.5 h. The tubes were permitted to cool to ambient temperature prior to, and vortexed vigorously following, each addition. Lastly, 4.6 mL of 0.22% ammonium molybdate and 0.2 mL Fiske-Subbarow reagent [20] were added. The Fiske-Subbarow reagent was prepared by mixing 40 mL 15% (w/v) sodium bisulfite, 0.2 g sodium sulfite, and 0.1 g 1-amino-4-naphtholsulfonic acid at ambient temperature for 30 min then filtering out undissolved solids. The tubes were then heated in a boiling water bath for 7 min, then quickly cooled in an ice water bath. The absorbance at 830 nm was recorded. Standards prepared from potassium phosphate dibasic in deionized water were subjected concurrently to the same procedure. Each sample was assayed for phosphorous in triplicate and the phospholipid content of the liposomes was determined from a calibration curve prepared from the standards analyzed in each run.

Bacteria growth and extraction

All bacterial strain sources and growth conditions were as described previously by Hartley [21, 22]. Briefly, *E.coli* SG12036-pIu121 was grown in 2% Lennox Broth (LB) with 30 μg/mL tetracycline. Five or six colonies were

inoculated into 5 mL growth broth and grown for 4 h with vigorous shaking (200–250 rpm) at 37 °C. A 250-μL aliquot of IPTG (50 mM) was then added and the culture was grown for another 30 min to an optical density of about 0.6 (at λ=600 nm). The *E coli* SG12036-pIU121 cells were centrifuged and the pellet was then treated with 100 μL lysozyme (0.4 mg/mL) dissolved in 10 mM Tris/1 mM EDTA buffer, pH 8.0 to digest the cell wall. After a 5- to – 10-min incubation period, the standard RNA extractions were carried out using a silica-guanidine thiocyanate protocol [23], provided as proprietary lysis buffer and isolation reagent modules in the RNeasy Total RNA kit (Qiagen). The standard bacterial protocol recommended by the manufacturer was followed. Total extracted RNA was quantified in a Beckman-Coulter spectrophotometer by measuring the absorbance at 260 nm (A_{260}) and calculating the total yield based on one unit of absorbance at A_{260} corresponding to 40 μg of RNA per mL of water. The concentration of RNA was used to determine the appropriate dilution for the NASBA reaction which was approximately 0.002 μg/μL. A series of 10-fold dilutions was made of this extracted RNA to determine the lowest amount detectable by the liposome-enhanced microtiter plate assay following NASBA. The purity of the nucleic acid was measured by making a 1:100 dilution in 10 mM Tris-HCl buffered to pH 7.5 and analyzing the A_{260}:A_{280} ratio.

Nucleic acid sequence-based amplification (NASBA)

NASBA primers were mixed to a final concentration of 0.2 μM in 15% (w/v) dimethylsulfoxide/1× NN buffer including 5 μL of the extracted RNA. NN buffer is composed of 40 mM ITP, 12 mM MgCl$_2$, 70 mM KCl, 5 mM DTT, 1 mM each dNTP, 2 mM each NTP, 1.5 mM ITP and 40 mM Tris. RNAse-free water and previously determined positive RNA extract from *E. coli* SG120368-pIU121 (0.01 μg RNA/5 μL, stored at −20 °C) were used as negative and positive controls, respectively. The mixture was heated at 65 °C for 5 min, then at 41 °C for 5 min. The three-enzyme cocktail (AMV RT, RNase H, and T7 RNAP, 5 μL) was added and the reaction was incubated at 41 °C for 90 min. The success of the reaction was confirmed by lateral flow assays developed by Hartley et al. [21] and further by the optimized microtiter plate sandwich-hybridization assay.

Sandwich-hybridization assay

Reacti-bind neutravidin-linked microtiter plates (Pierce Biotechnology, Inc., Rockford, IL) were washed with 2×200 μL wash buffer (composed of 0.05% (v/v) Tween-20, 0.01% bovine serum albumin (BSA)), followed by 200 μL 1× phosphate buffered saline (PBS). The manufacturer reported a binding capacity of 15 pmol per well. A 100-μL aliquot of biotinylated capture probe (0.1 μM in 50 mM potassium phosphate buffer, pH 7.5 containing 1 mM EDTA) was added and incubated for 30 min at 23 °C. Initially, capture probe concentrations ranging from 0.03 to 0.70 μM were applied. Unbound capture probe was removed, the wells were tapped dry, and washed thoroughly with 2×200 μL wash buffer, followed by 200 μL of previously optimized hybridization buffer [21] (9× sodium saline citrate (SSC) (1.35 M sodium chloride, 0.135 M sodium citrate, 0.01% sodium azide, pH 7.0), 30% formamide, 0.2% Ficoll 400 (Amersham Biosciences)). Synthetic DNA target solutions or NASBA products were prepared by dilution with hybridization buffer, applied as 100-μL volumes in triplicate wells, and incubated for 30 min. Unbound target solutions were removed and the plates washed thoroughly with 2×200 μL HEPES-saline-sucrose buffer. Reporter-probe-tagged liposomes (100 μL) were then added to the wells and incubated with the hybridized target for 30 min. Prior to the addition, the liposomes were prepared as follows:

a) For the concentration optimizations, 2.0-μm extruded liposomes (335±82-nm diameter) prepared with 0.013 mol% reporter probe were diluted to phospholipid concentrations of 0.025, 0.05, 0.1, 0.3, and 0.5 mmol/L (from Bartlett assay measurements) with HEPES-saline-sucrose buffer.

b) For the % reporter probe coverage optimizations, 0.6-μm extruded liposomes prepared with reporter probe coverage ranging from 0.0013–0.103 mol% were diluted to an optical density of 1.43±0.02 with HEPES-saline-sucrose buffer.

c) For the spacer length optimizations, 0.6 μm extruded liposomes prepared with 0.013 mol% reporter probe with a cholesteryl anchor and TEG, TEG+C$_3$H$_6$, TEG+(CH$_2$O)$_3$, TEG+C$_{12}$H$_{24}$, and TEG+(CH$_2$O)$_6$ were diluted to an optical density at 532 nm of 1.064±0.019 with HEPES-saline sucrose buffer.

d) For the diameter optimizations, 0.2-, 0.4-, 0.6-, and 2.0-μm extruded liposomes (201±29-nm, 274±47-nm, 308±53-nm, and 335±82-nm diameter) prepared with 0.013 mol% reporter probe were diluted to a phospholipid concentration of 0.1 mM with HEPES-saline-sucrose buffer.

In all cases, after the 30-min hybridization period, unbound liposomes were removed, the plates washed thoroughly with 2×200 μL HEPES-saline-sucrose buffer, and signal of bound intact liposomes read at λ_{ex}=540/35 nm, λ_{em}=590/25 nm in 100 μL HEPES-saline-sucrose. The supernatants were removed and wells diluted with 50 μL
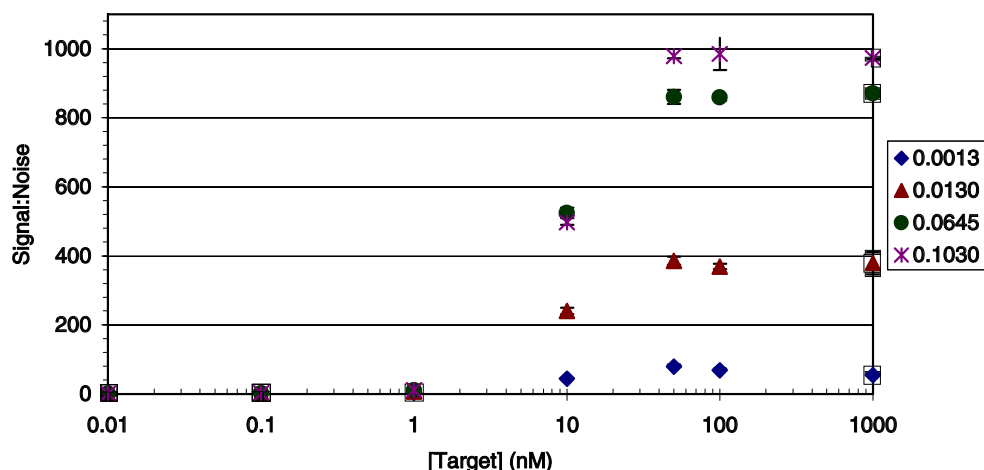
30 mM OG. After a 5-min incubation period, the fluorescence of the bound lysed liposomes was measured at λ_{ex}=540/35 nm, λ_{em}=590/25 nm.

For comparison purposes, in one experiment, a 5′-fluorescein-labeled reporter probe (2 μM) was used instead of reporter probe-tagged liposomes. After incubation for 30 min, the signal from hybridized probe was read at λ_{ex}=490/20 nm, λ_{em}=525/20 nm.

Results and discussion

Our goal was to optimize dye-encapsulating liposomes for use in a microtiter plate sandwich-hybridization assay for the detection of RNA from bacteria sources. For the optimization of this method, a 22-mer biotinylated capture probe was immobilized to neutravidin-coated microtiter plates and a 43-mer synthetic single-stranded DNA (ssDNA) target was permitted to hybridize prior to the addition of SRB-encapsulating liposomes which were tagged with a 22-mer reporter probe. Nanomolar range detection of the target was then achieved through surfactant-induced liposome lysis and release of encapsulated fluorescent dye molecules. Capture probe concentrations ranging from 0.03 to 0.70 μM were investigated yielding the optimal concentration of 0.1 μM. For the liposome optimization aspects of this work, the liposome size, reporter probe coverage, spacer length, and concentration were evaluated in terms of the optimal signal to noise ratio for the assay. For comparison purposes, a 3′-fluorescein-labeled reporter probe was used in lieu of dye-encapsulating liposomes to provide signal enhancement. Using the optimal conditions found, we applied this method for the detection of RNA amplified using NASBA. Where applicable, we compare this assay format to a lateral flow assay format using intact dye-encapsulating liposomes and the same probe and target sequences [3].

Optimal reporter probe coverage

The optimal concentration of reporter probe on the liposome surface was determined by incorporating various amounts of cholesteryl-TEG tagged DNA (0.0013–0.103 mol% of the total lipid input) into the liposome preparations, using them in microtiter plate sandwich-hybridizations, and measuring the signal upon liposome lysis. The mol% tag is expressed in terms of the initial phospholipid and cholesterol input, of which approximately 50% is expected to be on the exterior face of the lipid bilayer.

The optimal coverage of reporter probe had not yet been reached at 0.103 mol% over all target concentrations tested, since the overall signal to noise ratio continued to increase (Fig. 2) However, the rate of increase in the signal to noise ratio declined at higher coverages which indicated that a significant benefit towards further increases in probe coverage was unlikely. For example, the percentage increase in the signal to noise ratio when increasing the probe coverage from 0.0645% to 0.103 mol% was only about 3%, versus approximately 41% when the probe coverage was increased from 0.013% to 0.0645% at 1 nM synthetic DNA target.

In the previously reported lateral flow assays, the optimal coverage of cholesteryl-TEG reporter probe was found to be approximately 0.013 mol%, with a subsequent decline in signal at higher coverages [3]. In that format, the liposomes were preincubated with the target-containing solution for 10 min which allowed sufficient time for target molecules to hybridize with the liposomes. At higher probe concentrations, a decline in signal was observed, possibly due to the binding of multiple target molecules to a single liposome, which reduced the number of liposomes participating in the sandwich-hybridization assay. In the microtiter plate assay format described here, the liposomes were added only after all unbound target had been removed. Thus, there were fewer capture probe hybridized target

Fig. 2 Determination of the optimal reporter probe coverage on dye-encapsulating liposomes. Signal to noise ratios (y-axis) versus synthetic DNA target concentrations ranging from 0–1000 nM (x-axis) are shown for various liposome reporter probe coverages ranging from 0.0013–0.103 mol% (of the total lipid input) cholesteryl-TEG-anchored reporter probe. Analyses were done in triplicate and single-standard deviations are plotted

molecules available for interaction with the liposomes and consequently higher probe coverages on the liposome surface enhanced the opportunity for hybridization.

Optimal distance between lipid bilayer and reporter probe

The effect of spacer lengths between the liposome surface and the reporter probe was investigated with spacers composed of either straight chain hydrocarbons (C3 and C12) or poly(ethylene glycol)-based spacers (C9: $(CH_2O)_3$, C18: $(CH_2O)_6$) at a probe coverage of 0.013 mol% total lipid. These spacers were estimated to add lengths of 7.4 Å, 18.9 Å, 13.4 Å, and 24.2 Å, respectively, to the 24.3-Å TEG spacer already present on the cholesteryl anchor [24–26].

For all target concentrations tested, the maximum signal was obtained using the two longest hydrophilic spacer lengths of $(CH_2O)_3$ or $(CH_2O)_6$ (Fig. 3). However, liposomes prepared with the $(CH_2O)_3$ spacer yielded the highest signal to noise ratios, followed by $(CH_2O)_6$, C3, and TEG only (Table 2).

In addition, the signal to noise ratio of liposomes prepared with the $(CH_2O)_3$ spacer was 3.4 times that of those prepared with the C12 spacer at a synthetic DNA target concentration of 5 nM. We reasoned that the hydrophobic C12 spacer did not extend into the aqueous medium as much as its PEG-based counterparts and thus may have rendered the attached reporter probe less available to target sequences.

Increasing the length of the spacer in between the reporter probe and liposome surface was expected to allow the immobilized probe to interact with target molecules more freely and would more closely approximate solution phase hybridizations. One proposed mechanism suggests that single-stranded DNA is in close proximity to the

Table 2 Signal to noise ratios from liposomes prepared with varying spacer lengths

|	0.5 nM	5 nM	50 nM
TEG	2.6	123	417
$(CH_2)_{3+TEG}$	5.3	143	358
$(CH_2O)_{3+TEG}$	10.9	216	510
$(CH_2O)_{6+TEG}$	8.8	189	440

Data are analyzed from the experiment shown in Fig. 3. The top row lists the synthetic DNA target concentrations.

liposome surface, until hybridization with a complementary strand causes reorientation and extension into the surrounding medium [27]. In the previously reported lateral flow assay format where pre-incubation of liposomes with target molecules in the solution phase occurred, no dependence on spacer length was found [3]. In the microtiter plate assay format, the reporter probe can hybridize only to that target which has been hybridized to immobilized capture probe. Since the opportunities for hybridization in the microtiter plate format are more limited, the longer spacer length likely enhances the likelihood of binding. No information could be found in the literature regarding the effect of spacer length between liposomes and oligonucleotides, though the inclusion of a 6-carbon spacer between alkaline phosphatase and a 30-base reporter probe was reported to increase the signal to noise ratio in a sandwich-hybridization assay [28].

Optimization of liposome size and concentration

Liposomes of equivalent diameter were diluted to phospholipid concentrations of 0.025, 0.05, 0.1, 0.3, and 0.5 mmol/L prior to use in sandwich-hybridization assays. While the absolute fluorescence intensity increased with

Fig. 3 Effect of spacer length between cholesteryl-TEG anchor and reporter probe in the microtiter plate assay format. The spacer length (Å) (x-axis) is plotted against the fluorescence signal (y-axis, log scale) for 0.013 mol% reporter probe tagged liposomes which had been normalized to an O.D. of 1.064±0.019 and used for the analysis of 0–100 nM synthetic *atxA* DNA target on 0.1 pmol/μL biotinylated capture probe immobilized onto neutravidin microtiter plates. Analyses were done in triplicate and *error bars* represent one standard deviation
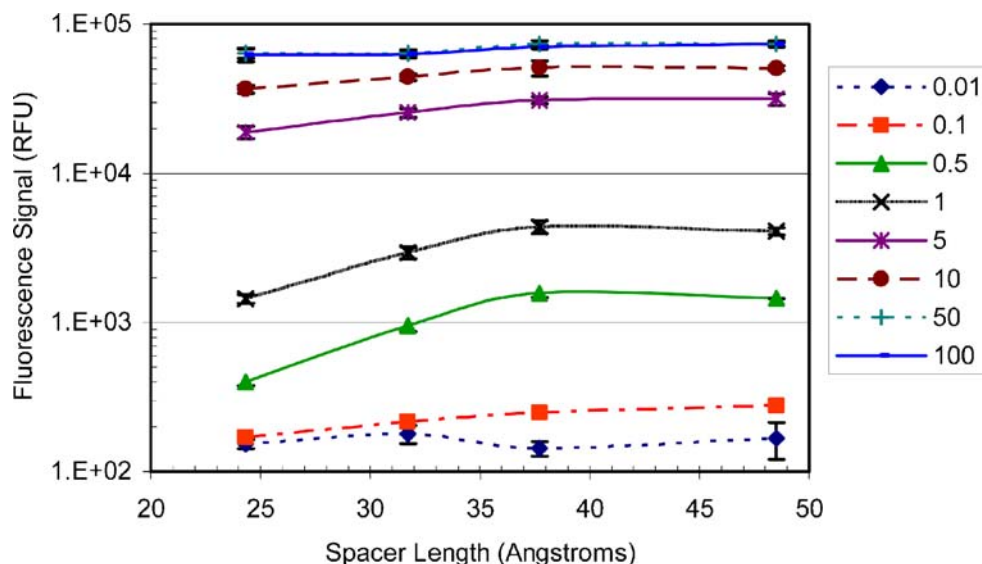
increasing phospholipid concentration, the background signal also increased. For example, for a concentration of 0.5 mmol/L phospholipid the background was approximately 4 times that of a concentration of 0.05 mmol/L phospholipid. Thus, for the concentrations tested, the maximum signal to noise ratio was obtained at a phospholipid concentration of 0.3 mmol/L (Fig. 4).

Using literature-reported values for the surface areas of composite lipids and the diameter determined through light scattering experiments, this correlates to approximately 2.01×10^{10} liposomes per assay. The estimated number of liposomes is based on the average diameter of the liposome population and does not take into account the width of the size distribution. Given the 0.013 mol% reporter probe coverage on the liposomes, approximately 3.6 pmol of reporter probe would be present per well (100-μL volume). This corresponds to 215 molecules of tag per 335-nm liposome, though only around half of this is available on the outer surface of the liposomes. This concentration of reporter probe is well above the range of detection for the sandwich-hybridization assays (~1 nM to 50 nM), thus additional liposomes do not add much benefit and instead run the risk of yielding higher background and non-specific signals in the absence of target [3]. In addition, the lowest variability was seen for this concentration of liposomes (CV≤4.8% at target concentrations above 1 nM).

The effect of liposome diameter on the microtiter plate assays was investigated by preparing liposomes of varying size by extrusion through 0.2-, 0.4-, 0.6-, and 2.0-μm-pore-size membranes resulting in liposomes with diameters between 200 and 335 nm. These liposomes were normalized to a phospholipid concentration of 0.1 mM prior to use in the sandwich-hybridization microtiter plate assays. The extrusion technique typically removes liposomes with diameters larger than the pore size of the membrane used for extrusion and serves thus to downsize the upper end of the population while reducing the degree of multilamellarity. Each of the diameters reported throughout this report is

the average of an assumed monomodal size distribution. The 2-μm extruded liposomes yielded a broad size distribution (335±82 nm), whereas 0.2-μm extruded liposomes yielded a much narrower size distribution (201±29 nm).

When normalized on the basis of phospholipid concentration, liposomes with a size distribution of 274±47 nm (extruded through 0.4-μm membranes) yielded the maximum fluorescence signal (Fig. 5). For a given phospholipid concentration, the number of liposomes present decreases with increasing liposome diameter. The signals observed at all target concentrations were similar for liposomes with an approximate diameter of 200 nm (ca. 1.9×10^{10} liposomes/well) to those liposomes with an approximate diameter of 310 nm (ca. 8.0×10^9 liposomes/well). Thus, the greater number of smaller liposomes did not overcome the increased volume of dye present in the larger population. However, when 335-nm liposomes were used (6.7×10^9 liposomes/well), a notable decline in signal was observed. This indicated that the benefit of increased internal volume was overcome by the decreased number of liposomes present or possible steric hindrance towards access to the hybridized target. With the LFAs, slightly larger liposomes (approximately 315-nm diameter) were found to be optimal [7]. The combination of solution-based and migration-based hybridization is apparently less affected by steric hindrance in that size range.

Assay performance and comparison of detection means

Using synthetic DNA target, a detection limit and upper limit for the assay of 0.5 nM and 50 nM were found, respectively (Fig. 6). The upper limit to this assay is likely a function of the limited binding capacity of the plates used in this assay. The coefficient of variation (CV) was ≤4.4% when target concentrations equal to and greater than 0.5 nM were considered. The high accuracy of the assay allows the discrimination of small concentration differences thus providing a highly sensitive assay, in which 1 nM differ-

Fig. 4 Effect of liposome concentration on the signal to noise ratio for various concentrations of synthetic DNA target. The number of liposomes per well (*x*-axis) was calculated on the basis of dynamic light scattering measurements and phospholipid concentration determinations. Each *point* is the average of triplicate determinations at each of the target concentrations tested and *error bars* represent one standard deviation
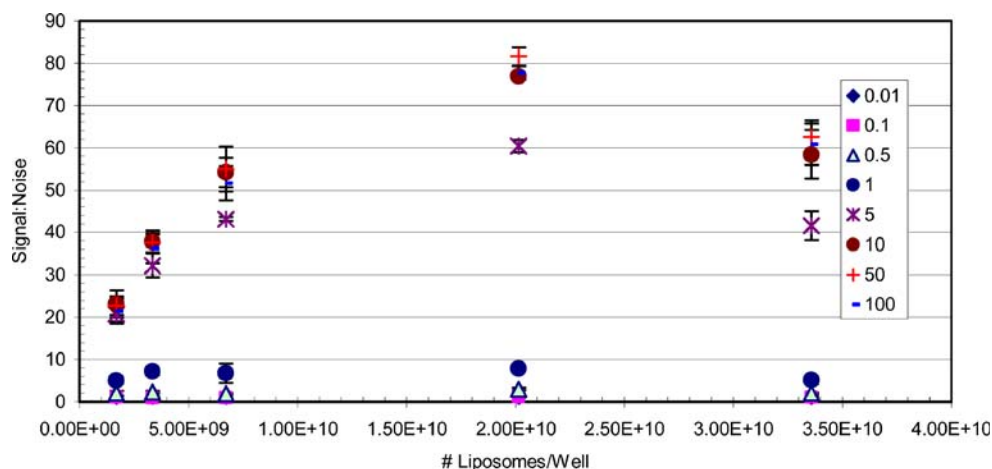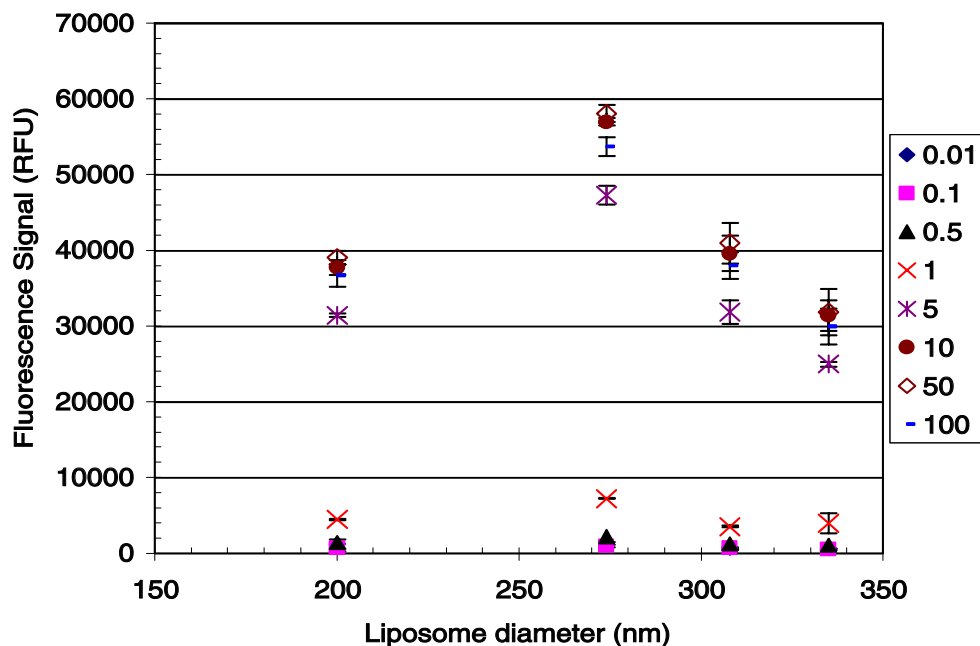
Fig. 5 Effect of liposome diameter (*x*-axis) on fluorescence signals (*y*-axis) for concentrations of synthetic DNA target ranging from 0–100 nM. Each *point* is the average of triplicate determinations with the *error bars* representing one standard deviation

ences in concentration between 0.5 and 20 nM can be determined.

The advantages of liposomes as signal amplification tools have been reported by many researchers who integrate these multi-label systems into analytical assays. Enhancements over single fluorophores ranging from 10-fold [29] to 1,000-fold [30] have been reported. Here, a comparison was made to a single-fluorescein-labeled reporter probe to demonstrate the utility of dye-encapsulating liposomes for sandwich-hybridization-based detection of nucleic acids. The signal enhancement stemming from intact versus lysed liposomes was also demonstrated (Fig. 7).

A signal to noise ratio of 985 was obtained for lysed liposomes versus 35 for intact liposomes and 1.8 for

fluorescein labeled reporter probe at a target concentration of 100 nM. At a 100-fold lower target concentration (1 nM), a signal to noise ratio of 11.2 was obtained for lysed liposomes versus 5.2 for intact liposomes and 1.3 for fluorescein-labeled reporter probe. Thus, when using the criteria of a signal to noise ratio equal to 3 for a limit of detection, the fluorescein-labeled probe could not be used to detect the target sequences even at a concentration of 100 nM. Over the synthetic DNA target concentrations tested, the minimum detectable target concentration for the fluorescein-labeled reporter probe was 50 nM, which resulted in a signal to noise ratio of 1.8. This signal to noise ratio equated to a target concentration of 0.1 nM when lysed liposomes were used, thus a 500-fold signal

Fig. 6 Effect of synthetic DNA target concentration (0–100 nM) on the fluorescence signal. Sulforhodamine B-encapsulating liposomes with the $(CH_2O)_3$ spacer normalized to a phospholipid concentration of 0.3 mM were used for these determinations. Bound liposomes were lysed with 50 μL 30 mM *n*-octyl-*β*-D-glucopyranoside. Each *point* is the average of 3 determinations at each of the target concentrations tested and the *error bars* represent one standard deviation
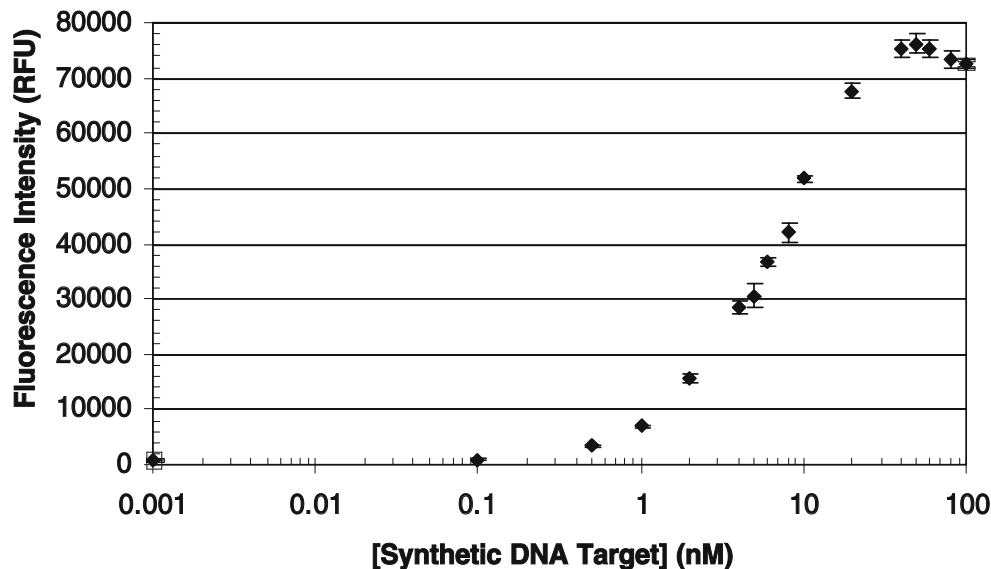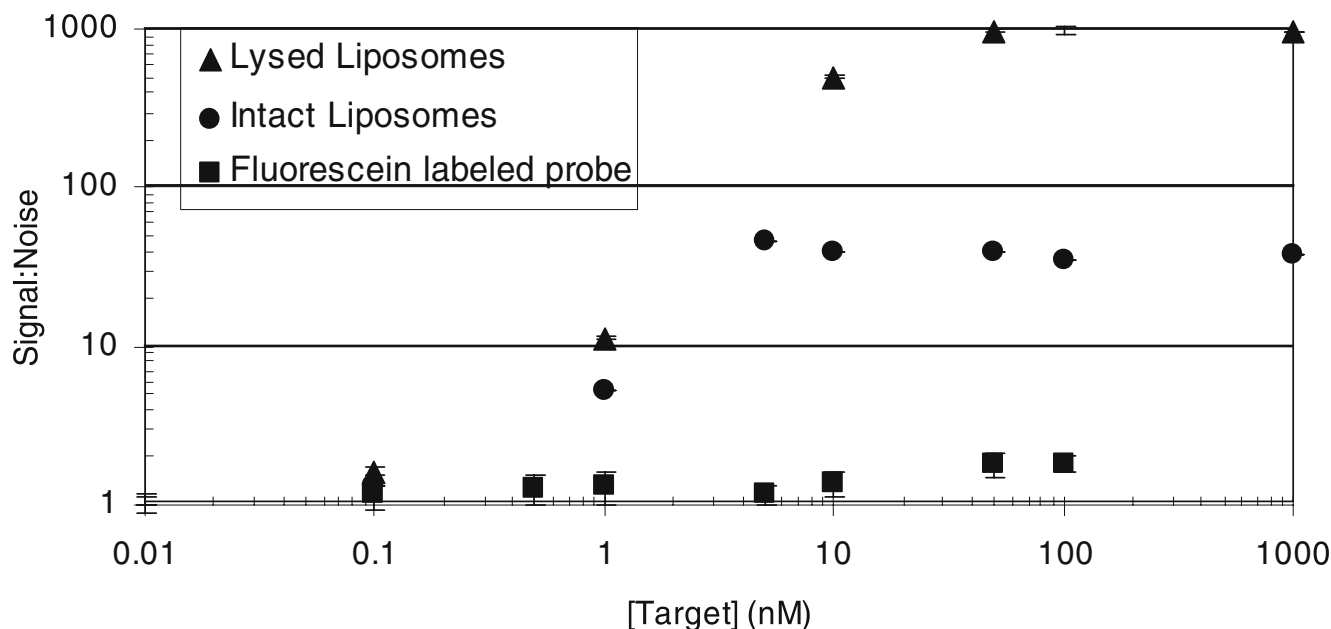
Fig. 7 Comparison of sandwich-hybridization-based detection of ssDNA (0–1000 nM) using fluorescein-labeled probe (■) to detection using both intact (●) and lysed (▲) dye-encapsulating liposomes. The liposomes used in these experiments were approximately 300-nm diameter, diluted to an optical density at 532 nm of 1.46, and had a reporter probe coverage of 0.103 mol% with the cholesteryl-TEG spacer. The signal to noise ratio (y-axis) of bound liposomes is shown before and after lysis with 50 μL 30 mM n-octyl-β-D-glucopyranoside. The fluorescein-labeled reporter probe was used at a concentration of 2 μM. Each *point* is the average of three determinations with *error bars* representing one standard deviation

enhancement was attained. When intact liposomes were used, the dynamic range of the assay was limited with a significant increase in the signal to noise ratio at 1 nM that reached a plateau by 10 nM synthetic DNA target. Sulforhodamine B dye undergoes fluorescence self-quenching when encapsulated at high concentrations within

liposomes, thus the fluorescence from intact liposomes is less than 3% of the fluorescence resulting from lysed liposomes. Lysis of liposomes releases the encapsulated dye and the subsequent dilution of the dye into the medium overcomes these self-quenching effects. However, it should be noted here that despite fluorescence detection of released

Fig. 8 Effect of extracted RNA amount prior to NASBA on the fluorescence signal. Liposomes with the $(CH_2O)_3$ spacer normalized to a phospholipid concentration of 0.3 mM were used for these determinations. Each *point* is the average of 3 determinations and the *error bars* represent one standard deviation
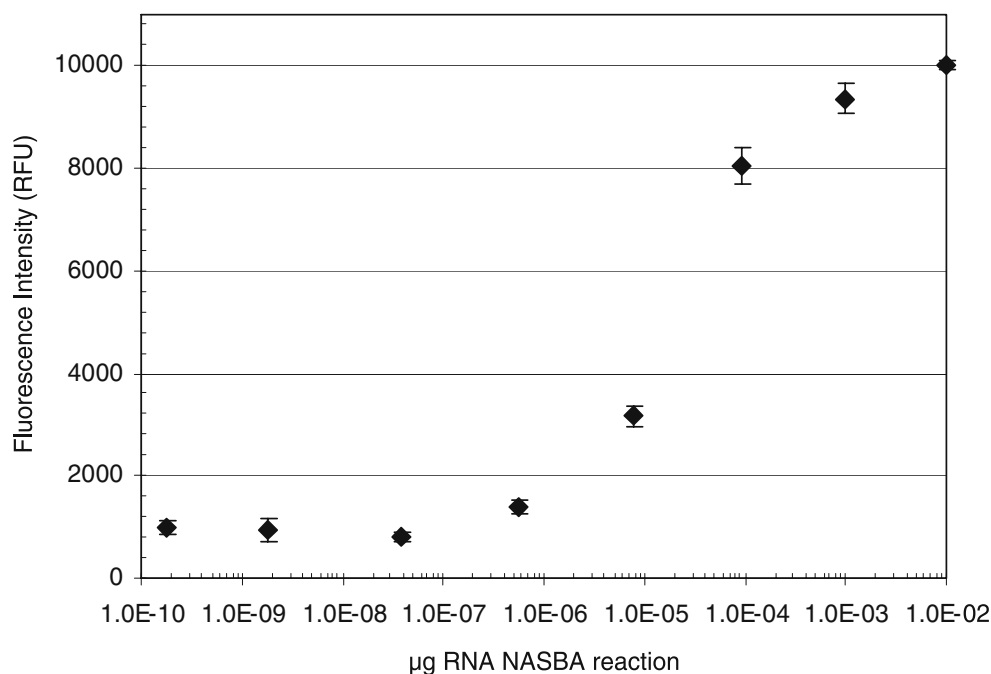
dye, the detection limit in the microtiter plate format was not significantly lower than that observed with intact dye-encapsulating liposomes in the lateral flow assay format [21]. In the LFA format, the LOD, defined as the negative control plus three times the standard deviation, was 1.5 nM. We attribute this to the limited diffusion available to both target and liposomes in the lateral flow assays versus in the wells of the microtiter plate. However, the lysis of liposomes here allows for a significant enhancement in the signal to noise ratio over the lateral flow assay format and in a thus more sensitive assay (i.e., at 100 nM target, the S:N=985 versus S:N=5.8, respectively [3]).

Detection of *atxA* mRNA amplified using NASBA

RNA samples from *E. coli* SG12036-pIU121, which over-expresses *atxA* mRNA, were extracted, amplified with NASBA and then detected in the microtiter plate format under optimal conditions in order to show that the method was not limited to synthetic DNA sequences. The samples were also analyzed using lateral-flow assays to verify that the extraction and NASBA reaction were successful [21]. A strong qualitative correlation of the microtiter plate assay and the lateral flow assays was found, i.e., all positive lateral flow assays were also positive using the microtiter plate format; all negative samples were also identified correctly to be negative using the microtiter plate format. For both assay formats, the minimum concentration of extracted total RNA required for a positive result was 1.1×10^{-7} μg/μL, which corresponded to 5.7×10^{-7} μg total RNA per NASBA reaction (Fig. 8).

However, it should be noted that the extracted RNA from the overexpression vector contains a much larger concentration of *atxA* sequences than would an extraction from *B. anthracis*, which produces few copies of *atxA* [21]

Conclusions

From this investigation and prior reports found in the literature, it has been clearly demonstrated that the use of liposomes as signal-enhancing reagents can lower the limit of detection when compared to singly tagged molecules. However, in many cases, the enhancement was not as significant as would be expected given the high internal capacity of liposomes. This has been attributed to two main factors: steric hindrance and multivalency. The relatively large diameter of liposomes has been suggested as a hindrance towards allowing multiple liposomes to bind to adjacent antigens [31, 32]. In addition, the kinetics of biorecognition element–liposome conjugate binding may be

limited by mass action due to their significantly lower diffusion coefficients. In this assay, an optimal liposome diameter of approximately 274 nm was found which likely was a balance between maximizing encapsulation volume and minimizing steric factors. Liposomes also have many biorecognition elements on their surfaces, thus one liposome can theoretically bind to several targets. While the signal enhancement stemming from a singly tagged fluorophore is one to one on a molar basis, a single liposome can bind to multiple antigens thus the relationship is not direct [32]. This multivalency was believed to be responsible for the higher association constant of antibody-labeled liposomes versus fluorophore-labeled antibodies [32–34]. We observed evidence of this multivalency effect previously in our work towards liposome optimization for lateral flow sandwich-hybridization assays [3]. However, this effect was less evident in the microtiter plate format, since liposomes were only introduced into the assay when specifically bound target sequence was present. An optimal reporter probe coverage of 0.103 mol% was found, resulting in approximately 215 reporter probe molecules per liposome with half of them expressed on the outside surface. An optimal phospholipid concentration of approximately 0.3 mM was found to yield the maximum signal to noise ratio. Using the optimizations, the advantage of using dye-encapsulating liposomes over single fluorophore labels was demonstrated, yielding a 500-fold signal enhancement for the detection of specific DNA oligonucleotide sequences. A limit of detection of 0.5 nM synthetic DNA target was found.

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