GM$_1$-functionalized liposomes in a microtiter plate assay for cholera toxin in *Vibrio cholerae* culture samples

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Received 9 January 2007
Available online 18 April 2007

Abstract

*Vibrio cholerae*, the causative agent for cholera, infects its host by expressing a protein consisting of two subunits: the pentameric cholera toxin B (CTB) and cholera toxin A (CTA). CTB frequently is used as an indicator of the presence of pathogenic *V. cholerae* and typically is detected using enzyme-linked immunosorbent assays (ELISAs). In lieu of an enzyme-linked detection method, we have developed GM$_1$ ganglioside-functionalized fluorescent dye-encapsulating liposomes for the detection of CTB produced by *V. cholerae* in a simple microtiter plate assay. Liposomes were compared with fluorescein-labeled antibodies and enzyme-linked secondary antibodies for quantification of purified CTB. A limit of detection for CTB using the liposomes was 340 pg/ml, which was comparable to that using the ELISA but 18 times lower than that using the fluorescein-labeled anti-CTB antibodies for the same purpose. The sensitivity of the assay provided by the liposomes was substantial, and the working range improved when compared with that of the fluorescein-labeled antibodies and the ELISA. In addition, the liposomes required shorter assay times, exhibited greater precision, and were less expensive compared with the ELISA. The liposomes were optimized with respect to phospholipid and ganglioside concentrations. The optimized liposomes were then used to probe culture supernatants from *V. cholerae* El Tor C6706 grown in Dulbecco’s modified Eagle’s medium and AKI medium for the presence of CTB.

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Keywords: CTB; Cholera toxin; Liposomes; ELISA; Fluorescence

Infection of the gastrointestinal tract by *Vibrio cholerae*, the causative agent of cholera, results in an estimated 100,000 to 200,000 deaths annually worldwide [1]. This disease not only affects impoverished areas but also poses a threat to the greater world population as a potential tool of bioterrorism [2,3]. *V. cholerae* infects its host by expressing a protein consisting of two subunits: the pentameric cholera toxin B (CTB, 58 kDa), consisting of 103 amino acids per mer, and cholera toxin A (CTA, 27 kDa), a disulfide-linked dimer [4]. Both subunits are required for infection, with CTB being responsible for targeting specific cellular receptors and CTA being an ADP-ribosyltransferase that disrupts G protein signaling. CTA acts by causing an increase in intracellular cyclic adenosine monophosphate (cAMP), leading to secretion of water and electrolytes [5]. The resulting cellular dehydration manifests as diarrhea and dehydration in the affected individual. CTB binds to the pentasaccharide chain of the GM$_1$ ganglioside, which is located on intestinal epithelial cells and other eukaryotic cell types [6]. Gangliosides are glycosphingolipids

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*Abbreviations used: CTB, cholera toxin B; CTA, cholera toxin A; cAMP, cyclic adenosine monophosphate; CT, cholera toxin; ELISA, enzyme-linked immunosorbent assay; SRB, sulforhodamine B; LOD, limit of detection; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] sodium salt; DPPE–SRB, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine-N-lissamine rhodamine B sulfonyl ammonium salt; HRP, horseradish peroxidase; HSS, Hepes–saline–sucrose; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; OG, n-octyl-b-D-glucopyranoside; S/N, signal/noise; PBS, phosphate-buffered saline; FlAb, fluorescein-labeled antibody; CV, coefficient of variance; HAb, HRP-conjugated anti-fluorescein antibody.

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doi:10.1016/j.ab.2007.04.019
that are natural receptors to various biological toxins, including Sendai virus [7], influenza virus [8], and botulinum, tetanus, and cholera toxins (CTs) [9–12]. They are composed of a hydrophobic ceramide portion and a hydrophilic oligosaccharide chain. GM\textsubscript{1} binds the pentameric B subunit of CT through both low-affinity monovalent interactions of B polypeptides with its carbohydrate moiety and high-affinity interactions of pentameric B polypeptides with multiple GM\textsubscript{1} molecules [13].

Owing to their intrinsic amphipathic nature, GM\textsubscript{1} and other gangliosides have been incorporated into lipid bilayers or liposomes as model membranes for the study of toxin interactions [14–16] and as biorecognition elements in several toxin sensors [17–19]. Liposomes are vesicles formed through the association of phospholipid molecules in an aqueous environment. The extrusion process used in their production serves to reduce multilamellarity to yield primarily unilamellar vesicles with large interior volumes. Some advantages of liposomes for analytical purposes include the ability to encapsulate hundreds to thousands of hydrophilic signaling molecules within their aqueous cores, the non-time-dependent release of encapsulant molecules, and the versatile conjugation of the lipid bilayer to various biorecognition elements, including the incorporation of lipophilic receptors that are otherwise difficult to use in biosensing [18,20,21].

Public health officials and cholera researchers alike have an ongoing need for improvements to cholera detection methodologies. As high-throughput technologies become the standard by which analysis is performed, protocol simplification can greatly benefit assay reliability and affordability. CT detection has been accomplished using radioimmunoassays [22], lateral flow assays [17], and latex agglutination assays [23,24], some of which are commercially available [23]. Of the currently available detection techniques, some of which are summarized in Table 1, perhaps the most commonly used is the enzyme-linked immunosorbent assay (ELISA) [25,26]. ELISAs typically are performed in microtiter plates, although bead-based ELISAs reportedly allow for CTB detection in the low picogram/milliliter range [27,28]. Sandwich immunoassays use either immobilized GM\textsubscript{1}, a soluble anti-CT antibody, and a secondary antibody–enzyme conjugate [26,29], the opposite formation where GM\textsubscript{1} is conjugated to the signaling molecule and the antibody is immobilized [18], or a dual antibody approach [30,31]. Enzyme-linked analyses can suffer from poor precision due to loss of activity on storage and require multiple and time-sensitive processing steps [32,33]. An alternative to ELISAs is the use of biorecognition element-tagged liposomes for direct detection of analytes without the need for enzymatic conversion of a substrate. The ganglioside-incorporating liposomes that have been used for biosensing purposes have used a fluorophore-tagged lipid [18] or enzyme conjugated to their bilayers [34] or have encapsulated a visible dye [17,19,35] or electrochemical marker [36] within their aqueous cores. However, none of these methods has been shown to be effective in analyzing samples of \textit{V. cholerae} culture for the presence of CT. Described here is a sandwich assay using novel GM\textsubscript{1} ganglioside and dye bilayer-functionalized dye-encapsulating liposomes that form a sandwich complex with CTB and a monoclonal anti-CTB antibody immobilized in a microtiter plate. The lipid bilayer of the liposomes was tagged with sulforhodamine B (SRB), and the interior volume also encapsulated SRB at a concentration of 150 mM. Although the bilayer tag aided in visualization, the release of the encapsulated fluorophores on surfactant-induced lysis was used to provide a low limit of detection (LOD) for CTB in the sandwich assay. SRB dye undergoes fluorescence self-quenching when encapsulated at high concentrations. Surfactant-induced lysis of the liposomes releases the encapsulated dye, and its subsequent dilution into the medium overcomes these self-quenching effects. An optimization of the assay based on liposome phospholipid content and ganglioside concentration was performed. Liposomes were compared with fluorescently labeled antibodies and with ELISAs for detection of purified CTB and were used to detect CTB in samples of \textit{V. cholerae} grown in two different media. This approach represents a significant improvement over other assays for CTB in its sensitivity, reproducibility, and cost-effectiveness.

## Materials and methods

### Materials

- 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC),
- 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)],
- sodium salt (DPPG),
- 1,2-dipalmitoyl-sn-glycero-3-phos-

### Table 1

<table>
<thead>
<tr>
<th>Assay format</th>
<th>LOD</th>
<th>Assay time</th>
<th>Detection method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic radioimmunoassay</td>
<td>0.1 ng/ml(10^{-14} g)</td>
<td>(~16\ h)</td>
<td>Liquid scintillation counting</td>
<td>[61]</td>
</tr>
<tr>
<td>ELISA (AP conjugate)</td>
<td>8 ng/ml</td>
<td>(~25.5\ h)</td>
<td>Spectrophotometer at 405 nm</td>
<td>[26]</td>
</tr>
<tr>
<td>ELISA (HRP conjugate)</td>
<td>15–20 ng/ml</td>
<td>(~5.5\ h)</td>
<td>Spectrophotometer at 450 nm and visual detection</td>
<td>[29]</td>
</tr>
<tr>
<td>Microtiter plate sandwich immunoassay</td>
<td>82.5 ng/ml(1.5 nM)</td>
<td>(~3.25\ h)</td>
<td>Fluorescence detection at $\lambda_\text{ex} = 550 \text{ nm}$, $\lambda_\text{em} \approx 580 \text{ nm}$</td>
<td>[18]</td>
</tr>
<tr>
<td>Latex agglutination assay</td>
<td>1–2 ng/ml</td>
<td>(~24\ h)</td>
<td>Visual detection</td>
<td>[23]</td>
</tr>
</tbody>
</table>

* The LOD has been converted to nanograms/milliliter from its original designation (in parentheses).
phatidylethanolamine-N-lissamine rhodamine B sulfonyl ammonium salt (DPPE–SRB), and the extrusion membranes were purchased from Avanti Polar Lipids (Alabaster, AL, USA). SRB was purchased from Molecular Probes (Eugene, OR, USA). The GM₁ ganglioside and CTB were purchased from Calbiochem (San Diego, CA, USA). The biotinylated and fluorescein-labeled anti-CTB antibodies were purchased from United States Biological (Swampscott, MA, USA), whereas the anti-fluorescein–horseradish peroxidase (HRP) conjugate was obtained from Rockland Immunochemicals (Gilbertsville, PA, USA). Culture media were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents used in these experiments were purchased from VWR (Bridgeport, NJ, USA). The liposome size distribution was determined by dynamic light scattering with a DynaPro LSR (Proterion, Piscataway, NJ, USA) using the Dynamics software program (version 6.3.01) and the Cumulants method of analysis [37,38]. Fluorescence measurements were made using a FLX800 microtiter plate reader (Bio-Tek Instruments, Winooski, VT, USA).

**Liposome preparation**

DPPC, DPPG, cholesterol, DPPE–SRB, and GM₁ ganglioside (40.9:20:1:51:7.0:0.40:0.28 μmol, respectively) were first dissolved in a solvent mixture containing 3 ml chloroform, 0.5 ml methanol, and 3 ml isopropyl ether and were sonicated for 1 min at level 6 in a bath sonicator (VWR Aquasonic model 150D) to ensure homogeneous mixing. A 45 °C solution of dye (2 ml of 150 mM SRB 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 a 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 of 150 mM SRB at 45 °C. The mixture was 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. The liposomes were then passed through a 20 × 1.7-cm column packed with Sephadex G-50 at approximately 4 ml/min using 1× Hepes–saline–sucrose (HSS) buffer (10 mM Hepes, 200 mM sodium chloride, 200 mM sucrose, and 0.01% sodium azide at pH 7.0). The liposome-containing fractions were then combined and dialyzed overnight against 1× HSS. Liposomes were also prepared with 0.01, 0.05, 0.2, and 0.5 mol% GM₁ ganglioside as well as dye-encapsulating liposomes with no ganglioside.

**Analysis of liposomes**

The phosphorus content for each liposome batch was determined using the Bartlett assay [39]. Liposome samples (20 μl) were dehydrated at 180 °C for 10 min and then mixed and heated with 1.5 ml of 3.33 N H₂SO₄ for 2 h at the same temperature. Then 100 μl 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 of the Fiske–Subbarow reagent [40] were added. The Fiske–Subbarow reagent was prepared by mixing 40 ml of 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 and then filtering out undissolved solids. The tubes were then heated in a boiling water bath for 7 min and then quickly cooled in an ice water bath. The absorbance at 830 nm was recorded using a Powerwave XL microtiter plate reader (Bio-Tek Instruments). Standards prepared from potassium phosphate dibasic in deionized water were subjected to the same procedure concurrently. Each sample was assayed for phosphorus in triplicate, and the phospholipid content of the liposomes was determined from a calibration curve prepared from the standards analyzed in each run. The total lipid concentration was calculated by multiplying the phospholipid concentration by the initial total lipid/phospholipid ratio.

The number of lipids per liposome (N_tot) was calculated as follows:

\[
N_{\text{tot}} = \frac{(\pi/a_L)\left(d^2 + (d - 2t)^2\right)}{L}
\]

where \(a_L\) is the average headgroup surface area per lipid, \(d\) is the hydrodynamic diameter from light scattering measurements, and \(t\) is the bilayer thickness [41]. The bilayer thickness was assumed to be 40 Å, and \(a_L\) was calculated using values of 71, 45, and 19 Å² for phosphatidylycerline, phosphatidylglycerol, and cholesterol, respectively [42,43]. Using these values and weighting by the mole fraction of each component, the \(a_L\) obtained for these liposomes was 47.9 Å²/lipid. The number of liposomes was then calculated by dividing the total lipid concentration by \(N_{\text{tot}}\). The number of GM₁ receptors per liposome was calculated by multiplying the molar fraction of each component by the total lipid concentration and then dividing by the concentration of liposomes. The number of dye molecules encapsulated per liposome was calculated using the equation of a sphere and the diameter from dynamic light scattering experiments under the assumptions of unilamellarity and a bilayer thickness of 40 Å and also assuming that the concentration of SRB present initially was that remaining within the liposomes after purification.

**Assay optimization conditions**

**Liposomes in analysis**

To test the efficacy of the liposomes for detecting CTB, a microtiter sandwich assay was developed. Reacti-bind neutravidin-linked microtiter plates (Pierce, Rockford, IL, USA) were washed with 3 × 200 μl wash buffer (composed of 0.05% [v/v] Tween 20, 0.01% bovine serum albumin [BSA], 20 mM Tris, and 150 mM sodium chloride, pH 7.0). Then 100 μl biotinylated anti-CTB antibody (10 μg/
ml in wash buffer) was added and incubated for 2 h at 23 °C. Unbound capture antibody was removed, and the wells were tapped dry and washed thoroughly with $3 \times 200 \mu l$ wash buffer. Standards composed of purified CTB in wash buffer, AKI medium or Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum, or supernatants from *V. cholerae* cultures grown in AKI or DMEM were diluted 1:1 in a wash buffer and incubated (100 μl/sample/well) in the anti-CTB conjugated plates at room temperature in the dark without shaking for 2 h. The plates were then washed twice with 200 μl wash buffer and once with 200 μl 1× HSS before applying 100 μl liposomes diluted in 1× HSS to a concentration of 10 μM phospholipid and incubating at room temperature in the dark for 1 h. Plates were then shaken for 10 min at 18 Hz in a fluorescence plate reader. Unbound liposomes were removed from the plates using 3 × 200 μl of 1× HSS. Intact bound liposomes were lysed with 50 μl of 30 mM n-octyl-β-D-glucopyranoside (OG) per well, and the fluorescence of each well was measured ($\lambda_{ex} = 540$ nm, $\lambda_{em} = 590$ nm). The data were fit using a five-parameter logistic:

$$y = b + \frac{a - b}{(1 + (\frac{x}{c})^d)}^e$$

where $a$ is the response at zero concentration, $b$ is the response at maximum concentration, $x$ is the CTB concentration, $c$ is the concentration yielding 50% response, $d$ is a slope factor, and $g$ is an asymmetry factor [44,45]. The LOD and quantitation were defined as the concentration equivalent to the background plus 3 SD and background plus 10 SD, respectively [46]. The background signal was the signal resulting from the above steps using wash buffer without CTB and was used in all signal/noise (S/N) ratio calculations.

Kinetic measurements were made using a microtitr plate prepared and incubated with 100 ng/ml CTB as described above. After washing away unbound CTB, dye-encapsulating liposomes with no ganglioside and liposomes with 0.01, 0.05, and 0.28 mol% ganglioside were diluted to a phospholipid concentration of 200 μM with 1× HSS. Then 100 μl of each liposome type was added to triplicate columns in the 96-well plate. After incubation for 5 to 120 min, the liposomes were removed sequentially from triplicate wells and the wells were washed with 3 × 200 μl of 1× HSS. Then 50 μl of 30 mM OG was added to all wells, and the fluorescence of the bound liposomes was measured ($\lambda_{ex} = 540$ nm, $\lambda_{em} = 590$ nm).

Fluorescein-labeled antibodies

The utility of fluorescein-labeled anti-CTB antibodies for the detection of CTB was completed as described above for liposomes with the following modifications. Standards of purified CTB (100 μl/well in triplicate) in wash buffer were incubated in the anti-CTB conjugated plates at room temperature in the dark without shaking for 2 h. The plates were then washed with 2 × 200 μl wash buffer, followed by 200 μl of 1× phosphate-buffered saline (PBS: 0.1 M phosphate buffer and 1.5 M sodium chloride at pH 7.0). Fluorescein-conjugated anti-CTB was diluted either 1:100, 1:171, 1:5000, 1:1000, or 1:10,000 in 1× PBS, and 100 μl per CTB-captured well was added and then incubated for 1 h at room temperature in the dark. The manufacturer’s suggested dilution for ELISAs was at least 1:10,000. Unbound antibodies were removed, and then the plates were washed with 3 × 200 μl of 1× PBS. The fluorescein-labeled antibody (FlAb) participating in the sandwich complex with CTB was read at $\lambda_{ex} = 490$ nm and $\lambda_{em} = 528$ nm.

Enzyme-linked immunosorbent assay

The ELISAs were completed as listed for the FlAbs, but clear neatavidin-linked plates were used rather than black plates. The microtitr plates used in all experiments were preblocked by the manufacturer with SuperBlock; thus, we opted to follow the manufacturer’s ELISA protocol without further blocking. HRP-anti-fluorescein F(Аb’)2 conjugate (goat) was diluted 1:4000, 1:6000, 1:8000, and 1:10,000 with 1× PBS. The manufacturer’s suggested dilution for ELISAs was between 1:2000 and 1:10,000. The diluted enzyme was then added to the sandwich complex formed between captured CTB and the FlAbs. The mixture was incubated for 30 min, and then the plates were washed three times with 200 μl of 1× PBS. TMB substrate solution (0.4 g/L) and peroxide solution (0.02% hydrogen peroxide in citric acid buffer) were mixed at equal volumes immediately prior to use (Pierce). Then 100 μl per well was added and incubated with shaking at 18 Hz for 10 min. The plate was then read at $\lambda = 490$ nm using an absorbance plate reader (PowerWave XS, Biotek Instruments).

Optimization of liposomes

To determine the optimal concentration for detection of CTB, liposomes containing 0.2 mol% ganglioside were diluted by their phospholipid content to 50, 10, 5, and 1 μM and were tested for their ability to detect purified CTB. Furthermore, performance of liposomes with various concentrations of GM1 ganglioside (0.01, 0.05, 0.2, 0.28, and 0.5 mol% of the total lipid input) incorporated into their bilayer was tested at a phospholipid concentration of 200 μM. Assessments were based on S/N ratios, LODs, and coefficients of variance (CVs).

Culture conditions

To determine the assay’s efficacy in detecting CTB from actual culture samples, *V. cholerae* El Tor C6707 was grown in triplicate (30 °C, 225 rpm) overnight in either AKI medium or DMEM (starting pH 7.4) to an OD$_{600}$ ≈ 1.2. Overnight cultures were diluted 1:1000 in fresh media and incubated (37 °C, 125 rpm) for up to 16 h. Aliquots of cells were taken at 3, 5, 7, and 16 h; measured for OD$_{600}$; and centrifuged (16,000 g, 10 min). The cell-free supernatants were mixed with the anti-protease cocktail (Pierce)
and stored at 4 °C for up to 13 h before being analyzed for CTB as described above.

**Results and discussion**

*Optimization of liposome sandwich immunoassay*

Our goal for this work was to develop a nonenzymatic assay for the detection of CTB in *V. cholerae* culture samples that would be an improvement over existing methods in terms of time, sensitivity, and cost. Toward this end, GM₁ ganglioside-functionalized fluorescent dye-encapsulating liposomes with a bilayer-embedded fluorescent dye tag were used as a detection tool in a 96-well microtiter plate sandwich assay. Vesicles containing dye have been used for CT detection recently in lateral flow assays [17] and flow injection analysis systems [35]; however, microtiter plate readers are more widely available and allow for high-throughput analysis.

Liposomes with a fluorescently labeled bilayer were compared with those containing 150 mM SRB along with a fluorescently labeled bilayer for the sandwich detection of CTB. The former species avoided dye leakage considerations, whereas the latter offered the potential for a lower LOD. CTB standard solutions were captured on anti-CTB-conjugated microtiter plates, followed by incubation of the captured CTB with GM₁-functionalized liposomes. Bound SRB-encapsulating liposomes were lysed with OG to provide an instantaneous signal enhancement, whereas bound bilayer-only tagged liposomes or intact dually tagged liposomes could be read directly. A calibration curve for CTB concentrations ranging from 0 to 10⁸ ng/ml using the dually labeled liposomes is shown in Fig. 1.

These data could be fit with excellent correlation ($R^2 > 0.999$) using a five-parameter logistic equation as is commonly used in immunoassays [44, 45].

The LOD for bilayer-only tagged liposomes was 1.14 ng/ml, as compared with 0.34 ng/ml for dually tagged liposomes after lysis (Table 2). The working range and CVs for lyzed dually tagged liposomes were 0.43 to 500 ng/ml CTB with a CV ≤ 3.7%, as compared with 3.9 to 250 ng/ml CTB with a CV ≤ 4.0% for bilayer-only tagged liposomes. This compares favorably with a prior report using liposomes incorporating 5 mol% GM₁ and 10 mol% rhodamine-tagged lipid for the sandwich microtiter plate analysis of CTB, where a minimum detectable concentration (defined as background plus 2 SD) of 1.5 nM (82.5 ng/ml) was found [18]. Here, using dually tagged and bilayer-only tagged liposomes with a 100-fold lower GM₁ content of the bilayer, minimum detectable concentrations of 0.32 and 0.78 ng/ml, respectively, were found using the same criteria. The effect of dye encapsulation was more notable in the enhanced S/N ratio; for lyzed dually tagged liposomes at a target concentration of 1000 ng/ml, the effect was 19 times that for intact dually tagged liposomes and 49 times that for bilayer-only tagged liposomes.

Although either liposome species could provide a sensitive and reproducible microtiter plate assay, the dual-label approach showed a higher S/N ratio at all target concentrations and also provides visualization capabilities in fluorescence microscopy [47]. Consequently, we further optimized these liposomes in terms of concentration and ganglioside coverage.

The concentration of liposomes was optimized using phospholipid concentrations ranging from 1 to 300 μM in terms of variability and effect on the S/N ratio. The phospholipid concentration can be correlated to the liposome concentration using dynamic light scattering data for the liposome diameter, assuming a unilamellar population of liposomes, using literature-reported values for lipid surface areas, and estimating the bilayer thickness to be 4 nm [42, 43], as outlined in Eq. (1) [41].

At low liposome concentrations (1 μM phospholipid corresponding to ~7.08 × 10⁶ liposomes), the signal reached a plateau at 100 to 500 ng/ml CTB and then declined with further increases in CTB concentration. This was not a function of exceeding the capacity of the capture antibody given that the signal increased further at the same CTB concentrations when higher concentrations of liposomes were used (5–300 μM phospholipid). Thus, the capacity of the liposomes at a phospholipid concentration of 1 μM had been reached at high concentrations of antibody-bound CTB (≥ 100 ng/ml). The S/N ratio increased with increasing liposome concentrations up to a phospholipid concentration of 10 μM, which yielded an S/N ratio of 179 at 300 ng/ml CTB (Fig. 2) and exhibited the lowest variability (CV ≤ 3.7%) at all CTB concentrations tested. This concentration of phospholipid, at an average liposome diameter of 350 ± 90 nm, corresponded to the presence of approximately 7.08 × 10⁶ liposomes and the
presence of approximately 37 nM ganglioside, presumably half of which is present on the exterior surface of the bilayer. This concentration of ganglioside exceeded the amount of CTB added approximately 1000-fold at a CTB concentration of 1 ng/ml and was equivalent to the added CTB concentration at 1000 ng/ml. Consequently, it was assumed that the upper end of the assay could be expanded by increasing the liposome concentration. However, with further increases in liposome concentration, the S/N ratio declined dramatically, largely a function of an increased background signal. A strong linear relationship ($R^2 > 0.997$) was observed between the liposome concentration (1–300 mM phospholipid) and the background signal obtained in the absence of CTB target. A strong linear relationship ($R^2 > 0.997$) was also observed between the fluorescence signal and liposome concentrations up to 10 μM phospholipid at all concentrations of target. Although the background continued to increase linearly with phospholipid concentrations greater than 10 μM, a corresponding increase in signal was not observed, thereby accounting for the decline in the S/N ratios.

We then prepared liposomes with GM1 ganglioside concentrations ranging from 0.01 to 0.50 mol% of the total lipid incorporated into the lipid bilayers and compared their signal enhancement in the sandwich assay. In these experiments, the amount of ganglioside available using a phospholipid concentration of 200 μM was equal to or exceeded the optimal amount found in the liposome concentration optimization experiments. For example, the same concentration of ganglioside found to be optimal previously (37 nM) was present here at a lower bilayer concentration (0.01 mol%) using a higher concentration of liposomes (200 mM) in the assay. A detailed discussion on multivalency and steric effects is beyond the scope of the current study but is the subject of ongoing investigations. At an equivalent liposome diameter and concentration, the S/N ratio increased up to a ganglioside coverage of 0.28 mol% and then declined at a coverage of 0.5 mol% (Fig. 3). Here the background signal was not correlated with the degree of ganglioside coverage. Thus, the decline in the S/N ratio at high ganglioside coverage may stem from the binding of a single liposome to CTB molecules bound to adjacent immobilized antibodies. At 0.28 mol% ganglioside coverage and 0.2 mM phospholipid, approximately $1.4 \times 10^{10}$ liposomes and 1034 nM ganglioside were present in the assay. Altering the ganglioside concentration between 0.05 and 0.50 mol% did not significantly affect the range of the assay; thus, an optimized coverage of 0.28 mol% is suggested. Overall, despite changes in phospholipid concentration ranging from 1 to 300 μM and variations in ganglioside coverage ranging from 0.01 to 0.50 mol% of

<table>
<thead>
<tr>
<th>Method</th>
<th>LOD** (ng/ml)</th>
<th>Working range** (ng/ml CTB)</th>
<th>Limits for CV in assay range (%)</th>
<th>Average assay time (h)</th>
<th>Relative cost (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dually tagged liposomes</td>
<td>0.34</td>
<td>0.43–500 (S/N = 1.2–178)</td>
<td>1.3–3.7</td>
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<tr>
<td>Bilayer-only tagged liposomes</td>
<td>1.14</td>
<td>3.9–250 (S/N = 1.4–3.6)</td>
<td>0.7–4.0</td>
<td>3.5</td>
<td>71</td>
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<td>FlAbs</td>
<td>6.19</td>
<td>14.3–250 (S/N = 1.6–3.6)</td>
<td>1.0–4.3</td>
<td>3.5</td>
<td>95</td>
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<tr>
<td>ELISA</td>
<td>0.49</td>
<td>4.7–300 (S/N = 1.3–1.5)</td>
<td>2.7–6.8</td>
<td>4.5</td>
<td>100</td>
</tr>
</tbody>
</table>

* LOD was defined as the concentration equivalent to the signal from the background plus 3 SD.

** The lower end of this range is defined as the limit of quantitation (i.e., the concentration equivalent to the signal from the background plus 10 SD). Values in parentheses are the S/N ratios at the ends of this range.

Relative cost was calculated by determining the total cost of the assay in terms of materials and normalizing by the most expensive technique to get a percentage of highest cost.

Fig. 2. Optimization of liposome concentration based on phospholipid content. S/N ratios were compared between various concentrations of liposome phospholipid content as determined by Bartlett assay [39]. Each point is the average of three determinations at each of the CTB concentrations tested (legend at right, ng/ml), and the error bars represent 1 SD.

Table 2
Comparison of Liposomes, FlAbs, and ELISA

the total lipid input, the robustness of the assay was evidenced by minimal variation in the CTB LOD (0.26–1.2 ng/ml).

The kinetics of binding to antibody-bound CTB was studied using liposomes with no ganglioside and liposomes with 0.01, 0.05, and 0.28 mol% GM1 ganglioside incorporated into their bilayers. In this experiment, CTB at a concentration of 100 ng/ml was introduced to an immobilized anti-CTB antibody. Liposomes normalized to a phospholipid concentration of 200 μM were permitted to bind and then removed sequentially over 2 h. The signal from those remaining bound at each time point is shown in Fig. 4. The kinetics is a function of both mass transfer from the bulk solution to the microtiter plate surface and binding of the liposomes to the captured CTB. Binding saturation by liposomes was reached by 45 min regardless of the concentration of ganglioside present (Fig. 4). Liposomes with no ganglioside present were used as a measure of nonspecific interactions and yielded only 0.5 to 2.1% (depending on the ganglioside concentration) of the signal obtained from liposomes. Allowing the incubation to proceed beyond 75 min resulted in an increase in nonspecific binding to 2.4 to 6.2% of the specific signal; thus, a maximum incubation period of 60 min is suggested. The lack of binding to liposomes without GM1 ganglioside showed the specificity of the signaling mechanism conferred by GM1. Although further specificity experiments were not completed in this work, others have shown a high degree of ganglioside specificity toward the respective biological toxins.

**Comparison with FIAbs and ELISA**

The performance of liposomes in this assay was compared with that in FIAbs and an ELISA for the detection of CTB. The bottom end of the assay (i.e., microtiter plate, biotinylated anti-CTB, and CTB) was the same for all detection means. In lieu of liposomes, FIAbs were added to form the upper layer of the analytical sandwich, followed by fluorescence detection at optimal wavelengths ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 528$ nm) of the bound species. For the ELISA, we used a secondary detection means through the incubation of HRP-conjugated anti-fluorescein antibodies (HAbs) with the bound FIAbs. Excess HAbs were removed, the HRP substrate was added to the plates, and absorbance was measured after incubation. The results from these studies are summarized in Table 2. The raw calibration data of fluorescence (FIAbs) or absorbance (ELISA) and the liposomes were found to be highly correlated ($R^2 > 0.97$) within the detection limits of the methods being compared. Of the three methods tested (liposomes, FIAbs, and ELISA), the maximum S/N ratio resulting from liposomes (S/N = 178) was significantly higher than that from either FIAbs (S/N = 3.6) or the ELISA (S/N = 1.5). Although several dilutions were tried, the optimal results reported for the latter methods used antibodies and antibody–enzyme conjugates, respectively, diluted to the manufacturer’s recommended levels for the ELISA. Using the criteria of background plus 3 SD, the LOD with liposomes (0.34 ng/ml) is comparable to that with the ELISA (0.49 ng/ml), but the former has a shorter assay time, lower variation, and an expanded range. These properties also are enhanced over the use of FIAbs, especially in terms of the LOD, which was 18 times lower than that for FIAbs (6.24 ng/ml CTB, 0.1 ml sample volume). The precision of the liposomes with triplicate samples was the lowest of the three methods (CVs = 1.3–3.7%) within their working range. The ELISA had CVs ranging from 2.1 to 6.8% within its working range from 4.7 to 300 ng/ml, whereas FIAbs exhibited CVs ranging from 1.0 to 4.3% within their working range from 14.3 to 250 ng/ml. A 30% savings in materials costs (phospholipid, cholesterol, and SRB vs. fluorescein-labeled anti-CTB, anti-fluorescein–HRP, and substrate) and a 1-h shorter assay period were realized through the use of liposomes instead of the ELISA.

Outside of the comparisons made here, the LOD for CTB detection using liposomes is equivalent to or better than that using most of the ELISAs and other methods.
outlined in Table 1. Ho and coworkers [17] and Ahn-Yoon and coworkers [35] reported lower LODs using flow injection analysis (10 ag/ml) and lateral flow assays (10 fg/ml), respectively, with dye-encapsulating liposomes alone than we observed here with dually tagged liposomes in the microtiter plate format. Our results consistently showed detection in the low nanogram/milliliter range and were comparable to those found by Singh and coworkers using a microtiter plate assay with bilayer-only tagged liposomes [18]. We postulate that the differences between these microtiter plate-based assays and the lateral flow assay/flow injection analysis system may stem from diffusion limitations in the microtiter plate wells given that other assay characteristics were similar. However, further investigation is of interest because a comparison of lateral flow assays with microtiter plate assays based on sandwich hybridization of DNA probes using dye-encapsulating liposomes yielded comparable detection limits for both techniques (1.5 vs. 6.0 fmol/assay, respectively) [48–50] and, similarly, the LOD using fluorescein-labeled probes with the microtiter plate format was comparable to that found with bead-based sequential injection analysis using the same principle (0.6 vs. 1.0 pmol/assay) [50,51].

Liposomes in analysis of CTB

Our intent here was to develop a method for quantitative laboratory-based measurements of CT expression in V. cholerae cultures that would provide a substitute for ELISAs and find its application in CT virulence expression studies. CTB was considered to be an acceptable model analyte for CT due to the requirement of CTB for assembly of functional CT dimer (1 CTB pentamer per functional CT unit). For our purposes, we were concerned only with CTB production, although this assay could be readily adapted for the definitive detection of the holotoxin through the use of a CTA-specific capture antibody. We tested the efficacy of the liposome assay for detecting CTB from cultures of V. cholerae by sampling supernatants from cultures and measuring the content against standards of purified CTB in culture media. Although liposome-based methods have been used previously in other formats for the detection of CTB standards in buffer or in spiked water samples [17,35], their application in growth media has not been shown. The range of the assay found using standards in buffer was believed to be sufficient based on literature-reported values for CT produced in cell culture. For example, in culture supernatants, 11.0 nM CTB (0.94 µg/ml) was observed after overnight growth of V. cholerae P27459 at 37 °C in AKI medium [52], approximately 110 ng/ml CTB was observed after growth of V. cholerae C7258 in AKI [53], and 15.8 nM CTB (1.34 µg/ml) was observed after 4 h growth of V. cholerae O395 at 37 °C in the T84 human epithelial cell medium [52]. We studied the effect of the culture media on the assay through comparing the LOD, S/N ratio, and variability of the fluorescence signal using CTB standards diluted in wash buffer with those diluted 1:1 in wash buffer and media [52]. The culture media present in the initial incubation of CTB standards with immobilized antibody did significantly affect the S/N ratio of the lysed liposomes. For example, the background signal in the absence of CTB in AKI was approximately 3.9 times less than that observed in DMEM. However, overall the presence of either DMEM or AKI in a 1:1 dilution in wash buffer did not significantly affect the performance of the assay. When compared with wash buffer alone, the LOD was similar, the assay range was equivalent, and the CV was low (≤6.1%) throughout the range of the assay for CTB standards in the presence of these culture media. Given the demonstrated robustness of the method, we would anticipate that this method could be applied to environmental or clinical isolates for monitoring environmental occurrences or epidemics of toxigenic V. cholerae. Appropriate food, environmental, and clinical sample preparation techniques, such as those used by Ligler and coworkers, could be applied to expand the utility of the method [54]. In addition, through the encapsulation of different fluorescent dyes and reliance on the specificity of other gangliosides as recognition elements, this method could be adapted for multianalyte detection [18].

Once the effectiveness of this method in culture media had been established, we used the method to monitor CT production by V. cholerae after 3, 4, 7, and 16 h following inoculation into fresh media. We examined the production of CT in both DMEM and AKI medium because we had determined that both of these media could support the growth of Caco-2 epithelial cells that are being used to model V. cholerae infection kinetics in ongoing work [47]. CTB standards prepared in each
medium were run to allow for quantitative results. Although CT was clearly present in AKI-cultured *V. cholerae* (Fig. 5), its presence in DMEM was considerably lower and more variable. The lower CTB production in DMEM was not entirely surprising given that the cultures also exhibited lower growth in DMEM than in AKI. A final cell density for DMEM-grown cells was only 37% of the final cell density of the AKI-grown cells in AKI. The cultures also exhibited lower growth in DMEM than in AKI. A final cell density for DMEM-grown cells was only 37% of the final cell density of the AKI-grown cells after 16 h. The results indicate that only AKI is sufficient for making CT (as measured though CT binding to the GM1 ganglioside) under uncontrolled (pH, O2, and redox) conditions. This is the first time, to our knowledge, that these media were compared for production of CT by *V. cholerae*. However, the superiority of AKI was not unexpected given that this medium was developed specifically for *V. cholerae* growth and CT production [55–58]. It was apparent that the production of CT in AKI increased over 7 h but then diminished considerably as the cultures grew overnight (16 h). We have attributed this to pH changes in the medium between 7 and 16 h. Although the initial pH was 7.4, the pH in the AKI medium dropped to less than 6.0 after 16 h, a pH level that has been found to be too low for CT synthesis (J. C. March, unpublished results). Furthermore, prolonged growth, cell lysis, and subsequent protease release likely resulted in proteolysis of any accumulated CTB. The technical errors for these data are comparable to those seen for the CTB standards (CVs = 1.2–5.8), thereby demonstrating the precision of the method. We will use these results in the development of a cell culture model for *V. cholerae* infection of epithelial cells.

**Conclusions**

We have demonstrated a high-throughput technique involving fluorescently labeled dye-encapsulating liposomes for the detection of CT. We optimized the concentration of the liposomes to 10 μM phospholipid and the amount of ganglioside in their bilayers to 0.28 mol% of the total lipid input. Comparison of the liposomes with the more commonly used method of ELISAs demonstrated a comparable limit of detection and lower processing time for liposomes (Table 2). Dye-encapsulating liposomes have been shown previously to yield substantially lower limits of detection than the use of fluorophore-labeled reporter probes [59] or antibodies [60]. Liposomes were more sensitive than FIAbs and were calculated to be 25 to 30% less expensive than FIAbs and ELISAs. We then used them to probe samples from cultures of *V. cholerae* for CTB and found that only AKI medium (and not DMEM) was suitable for CTB production under the uncontrolled (pH, O2, and redox) conditions investigated.

Although the goal of this study was to develop a quantitative laboratory method for monitoring CTB expression and kinetics of transport, we anticipate that this assay could also be modified for use in clinical or environmental isolates suspected to contain CTB. The low LOD, robustness, and minimal variation of the liposome assay could save public health officials valuable time in preventing an outbreak of cholera.

**Acknowledgments**

We thank Ronald Taylor of Dartmouth Medical School for provision of *V. cholerae* El Tor C6707 and thank Antje Baumner of Cornell University for use of the rotary evaporator and analytical equipment. We also thank Faping Duan for help with *V. cholerae* culturing. This work was supported by the Life Sciences Initiative at Cornell University.

**References**


