Appl Biochem Biotechnol (2011) 165:548–558
DOI 10.1007/s12010-011-9274-3

Detection of Liposome Lysis Utilizing an Enzyme–Substrate System

Daniel J. Wichelecki · Trisha M. McNew · Aysegul Aygun · Kathryn Torrey · Larry D. Stephenson

Received: 13 July 2010 / Accepted: 28 April 2011 /
Published online: 24 May 2011

Abstract A novel optical reporter system was developed to verify encapsulation and subsequent release of a foreign molecule in liposomes. The protocol utilizes a single enzyme and substrate. We encapsulate *o*-nitrophenyl-β,D-galactopyranoside (ONPG) and measure its release by detecting the levels of *o*-nitrophenol created when the encapsulated ONPG is released and hydrolyzed by β-galactosidase. Using this method, liposome formation and subsequent lysis with Triton X-100 were verified. This new protocol eliminates the complications of multiple reaction enzyme detection methods, along with the chance for false negatives and unreliable data seen when using fluorescent particles as reporters.

Keywords β-Galactosidase · Encapsulation · Liposome · Lysis · ONP · ONPG

Abbreviations

β-gal	β-Galactosidase
DMPC	1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine
DTT	Dithreitol
ESEM	Environmental scanning electron microscope
G-6PD	Glucose-6-phosphate dehydrogenase magnesium acetate
LMV	Large multilamellar vesicle
MLVs	Multilamellar vesicles
ONP	*o*-Nitrophenol
ONPG	*o*-Nitrophenyl-β,D-galactopyranoside
PBS	Phosphate-buffered saline
SUV	Small unilamellar vesicles

D. J. Wichelecki · T. M. McNew · K. Torrey
Oak Ridge Institute for Science and Education, Oak Ridge, TN, USA

A. Aygun · K. Torrey · L. D. Stephenson (✉)
Construction Engineering Research Laboratory (CERL), U.S. Army Engineer Research and Development Center (ERDC), Champaign, IL 61822, USA
e-mail: Larry.D.Stephenson@usace.army.mil

A. Aygun
The Pertan Group, 44 Main Street, Suite 403, Champaign, IL 61820, USA

Introduction

Bangham et al. first discovered liposomes almost 50 years ago [1]. Since then, liposomes have been used in drug delivery, nucleotide delivery, gene therapy, and as models for artificial cells [2–4]. They have also been utilized to transmit dyes to textiles [5], pesticides to plants [6], enzymes and nutritional supplements to foods [4], and cosmetics to the skin [4, 7].

During liposomal encapsulation of a solution, dried lipid films swell in an aqueous solvent, and the amphipathic lipids align based on hydrophobicity to form multilamellar vesicles (MLVs) [1, 8]. When encapsulating a hydrophilic compound, it is important to verify that liposome formation does occur. Many experiments use various forms of chromophores, fluorophores, or fluorescent nanoparticles in the hydration solution to verify enclosure based on spectrophotometric or fluorometric readings of the internalized reporter before and after release [9–12]. An increase in emission intensity would indicate the escape of internalized material and consequently, liposomal lysis.

As with most methods, there are a few limitations. For instance, upon excitation, chromo/fluorophores emit continuously and are thus deemed "always on," which can lead to false negatives. The most significant example is that fluorescent nanoparticles are so bright that they can shine through the liposomes [9]. Therefore, there is no significant difference in fluorescent detection before and after lysis regardless of actual release. This phenomenon prompts one to consider whether it is equally likely that chromophores or fluorophores with comparable quantum yields may partially, if not fully, shine through as well.

Furthermore, many chromo/fluorophores have poor photostability, are susceptible to photobleaching [13, 14], and can exhibit extensive blinking. Photobleaching and poor photostability can lead to false negatives and inaccurate standard curves or blanks. This is particularly disadvantageous if photoinduction is used as the mechanism for lysing the liposomes. Additionally, blinking can lead to lowered accuracy of spectral readings.

To eliminate the inaccuracies of current fluorescent detection methods, a novel detection method was devised by means of an enzyme–substrate-based colorimetric assay utilizing β-galactosidase (β-gal) and *o*-nitrophenyl-β,D-galactopyranoside (ONPG). β-Galactosidase is an enzyme responsible for lactose metabolism in *Escherichia coli* in which it hydrolyzes lactose into its monosaccharide units. ONPG is a lactose analog, which when cleaved by β-gal produces *o*-nitrophenol (ONP) and galactose [15] (Fig. 1). ONP absorbs light at a wavelength of 420 nm. Therefore, if liposomes loaded with ONPG are lysed with a detergent [16], lysis can be detected by adding β-gal to the lysant supernatant and recording the increase in absorbance at 420 nm (Fig. 2).

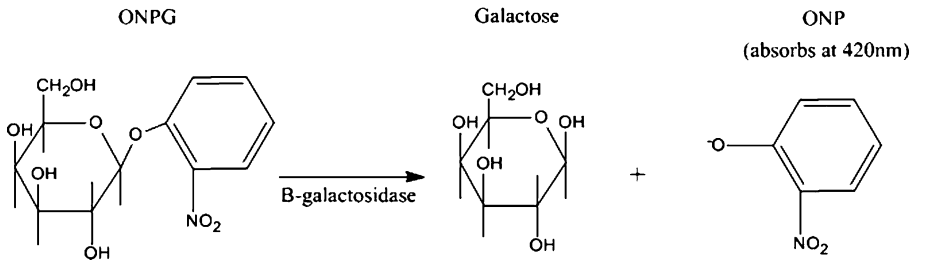

Fig. 1 β-Galactosidase (β-gal) hydrolyzes *o*-nitrophenyl-β,D-galactopyranoside (ONPG) into galactose and *o*-nitrophenol (ONP) which absorbs light at 420 nm in alkaline conditions. At 25 U/mL, β-gal takes 2 min to react completely

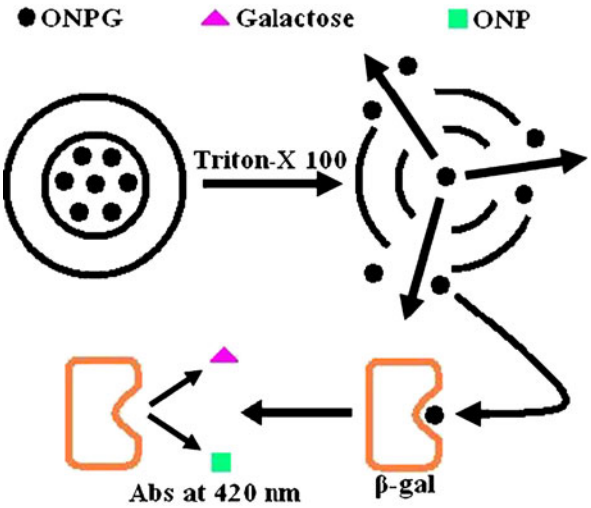

Fig. 2 ONPG is encapsulated inside the liposomes. Triton X-100 lyses the liposomes and releases the ONPG. β-Gal then cleaves ONPG into ONP (absorbance at 420 nm) and galactose

The only other enzymatic assay for liposomal release was found in Kinsky et al. [17]. Their method requires hexokinase, G-6PD, ATP, $NADP^{+}$, and Mg^{++}. There are more variables to consider, and therefore, more room for error in this method. This new method has the advantage of simplicity.

Our method greatly improves the accuracy of detection of liposome lysis. Since ONP is significantly more photostable than common chromo/fluorophores, the standard curve will be more accurate for determining how much ONP was created, and thus how much ONPG was released. Also, due to the innate nature of ONPG, the detection method will not be hindered if light is shined on the liposomes for photoinduced lysis. The ONP will not be affected by photolysis because it is not formed until after photoinduction is complete and β-gal is added. In this case, it eliminates the chance of false negatives. Furthermore, the assay is constituently in the "off" state until lysis occurs. Therefore, with a proper blank, no false negatives can occur as is the case with fluorescent reporters that are constantly in the "on" state (as mentioned previously).

Materials and Methods

Reagents

Chloroform, dithreitol (DTT), NaCl, Triton X-100, Tris, ONPG, and lyophilized β-gal were purchased from Sigma-Aldrich (St. Louis, MO). The 10× phosphate-buffered saline (PBS) solution was purchased from Fisher Scientific (Pittsburgh, PA). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL).

Enzyme Preparation (Dilution and Storage)

The lyophilized β-gal (8.04 mg) was suspended and stored in 1 mL of 20% *v*/*v* glycerol and 4 mL of Storage Buffer (50 mM Tris, 100 mM NaCl, 0.2 mM DTT, pH 8.0). The

suspension of the protein gave 1,447.2 U (the amount of enzyme needed to catalyze 1 μmol of substrate to product) per milliliter. Aliquots of 100 μL were dispensed into 50 chilled Eppendorf tubes and stored at −20 °C. The stock solution of β-gal was diluted to 25 U/mL (2.15 mg/mL) for use in the experiment.

ONPG Standard Curve

A standard curve was developed to determine the correlation between moles of ONP and absorption at 420 nm (A_{420}). Eight hundred microliter of 0.08 M PBS was mixed with 100 μL of 25 U/mL β-gal in a disposable cuvette. A_{420} readings were then taken after 0, 50, 100, 150, 200, and 250 nmol of ONPG (added in 20-μL fractions of 2.5 mM ONPG) was added to the solution using a Cary 50 UV-visible spectrophotomer (Varian, Palo Alto, CA). Three minutes was allowed for the reactions to complete before the absorbance readings were taken. Aliquots (50 nmol) of ONPG were added to the same solution after each reading until a total of 250 nmol of ONPG had been added. This was blanked with 0.08 M PBS, pH 7.7.

Lipid Film Preparation

The lipid films were prepared by dispensing 37.5 mg DMPC into a 3-mL glass Eppendorf tube. Then, 1.5 mL of chloroform was added to dissolve the DMPC into a homogenous solution. The solution was vortexed until all DMPC was dissolved. The DMPC/chloroform solution was dried in a Savant SC 110 Speed Vac (GMI, Ramsey, Minnesota). The drying speed was on high, the centrifuge was off, and the vacuum was on. Lipids were allowed to dry overnight into a thin film on the glass Eppendorf tube.

Liposome Preparation

Once completely dry, the lipid film was suspended in a warm (5 min in 45 °C water bath) solution made up of 4 mL of 0.08 M PBS of pH 7.7 and 1 mL of 2.5 mM ONPG. The suspension was transferred into a 15-mL conical tube with a 2.5-mm magnetic stir bar and set to hydrate in a 45 °C water bath, spinning at 1,100 rpm. The hydration solution was removed from the bath and vortexed every 5 min for 1 h, 5 s at a time. After this step, the lipid solution was milky white. Once hydration was complete, the suspension was subjected to three freeze–thaw cycles using liquid nitrogen.

Liposome Wash Step

The post-freeze–thaw liposomes were dispensed in 1-mL aliquots into five Eppendorf tubes and then centrifuged with an accuSpin Micro 12R centrifuge (Fisher Scientific) at $13.3 \times 1{,}000\ \text{min}^{-1}$ for 20 min. After centrifugation, the supernatant was removed, and each pellet was resuspended in 1 mL of 0.08 M PBS, pH 7.7. The resulting resuspensions were then combined in a test tube and gently mixed with a pipette to homogenize the mixture.

Liposome Lysis and Detection

Two Eppendorf tubes were filled with 1 ml of homogenized liposome solution. Then, 50 μl of 1.0% Triton X-100 was added to one of the Eppendorf tubes. One was left as a blank. Both tubes were vortexed and incubated for 15 min (vortexing every 90 s for the duration).

After incubation, 100 μL of 25 U/mL β-gal was added to each Eppendorf tube and left to react for 3 min. The mixtures were then centrifuged at $13.3 \times 1{,}000$ min^{-1} for 20 min, and the supernatant was read by a Cary 50 UV-visible spectrophotometer. The blank was zeroed at A_{420}.

Timed Triton X-100 Experiments

Liposomes were formed as previously described. From the homogenous solution, five Eppendorf tubes were filled with 1 ml of solution. Then, 50 μl of Triton X-100 was added to one tube. Every 5 min, 50 μl of Triton X-100 was added to another tube (repeated three more times). Samples were vortexed every 90 s. This was done such that after 20 min, there are four tubes which have been incubating in Triton X-100 for 20 min, 15 min, 10 min, and 5 min, respectively. Then, β-gal was added to all five tubes and allowed to react for 3 min. The absorbance at 420 nm was then read and recorded (blanked with 0.08 M PBS, pH 7.7).

Liposome Micrographs

Micrographs of the liposome solution were taken after the freeze–thaw step and after membrane disruption with Triton X-100. Liposomes were visualized with phase contrast microscopy (PCM) using an Eclipse E400 (Nikon, Melville, NY) and a scanning electron microscopy (SEM) using Jeol JSM 6390 (Jeol Ltd., Japan).

The PCM images were captured using a SPOT Insight 2 MP Firewire Color Mosaic (Diagnostic Instruments, Inc.; Sterling Heights, MI). Pictures were taken using ×400 and ×1,000 magnification.

The SEM was employed to visualize morphological changes before and after lysis of the liposomes. To prepare SEM samples, a drop of the liposome solution was placed on a double-sided tape attached on glass slide and was frozen at −80 °C for approximately 24 h. The frozen liposomes then lyophilized (Virtis Model Benchtop K, USA) at −40 °C for approximately 48 h. Samples were coated with a thin layer of gold before SEM analysis.

Buffer Control

The absorbance of 1 mL 1.0% Triton X-100 at 420 nm was found using the spectrophotometer. The samples were blanked with 0.08 M PBS, pH 7.7.

Lipid Contribution Control

Liposomes were prepared according to the procedure outlined previously, except the hydration solution did not contain any ONPG. They were hydrated in 5 mL 0.08 M PBS, pH 7.7. After freeze-thawing, the wash step was skipped, and the lysis protocol began. The liposomes were lysed and had their absorbance read as previously described.

Detergent's Effect on β-gal

Two Eppendorf tubes were filled with 50 nmol ONPG and 800 μL 0.08 M PBS, pH 7.7. Fifty microliters of 1.0% Triton X-100 was added to one tube and allowed to incubate for 15 min, vortexing every 90 s. Then, 100 μL of 25 U/mL β-gal was added to both tubes and allowed to react until completion (3 min). The absorbance values were taken at 420 nm using the spectrophotometer and blanked with 0.08 M PBS, pH 7.7.

Results

Visualization of Liposomes

As shown in the optical micrograph in Fig. 3, there is a marked difference between the structures of the LMV liposomes before and after the addition of the lysant detergent. Figure 3a, c was taken after the freeze/thaw step of liposome hydration. The liposomes are of many different sizes and evenly dispersed in solution. The smaller liposomes are at different depths and therefore appear blurry in these images. It should be noted that only liposomes over 500 nm in diameter are detectable by optical microscopy [18].

Figure 3b, d was taken 15 min after 1.0% Triton X-100 was added (vortexing every 90 s). In this picture, the liposomes are clumped up into large aggregates. Also, there is some liposome swelling and disruption into fragments too small to be seen; hence, the large "open" areas. In Fig. 4, a SEM micrograph of liposomes after the freeze–thaw step is shown in the inset, while the disrupted liposomes are shown in the main image. From the figure, swelling and disruption of the membrane are clearly seen.

These results are most likely a side effect of osmotic lysis. When the membranes are disrupted, fluid rushes in to equilibrate the solute concentrations and causes the liposomes to either swell or explode. Anderson and Krinsky [19] describe a similar phenomenon while achieving liposomal release with 1.0% Triton X-100.

Such significant changes in dispersion caused by detergent addition and vortexing indicate membrane disruption. Because of the wash step, the concentration of ONPG is

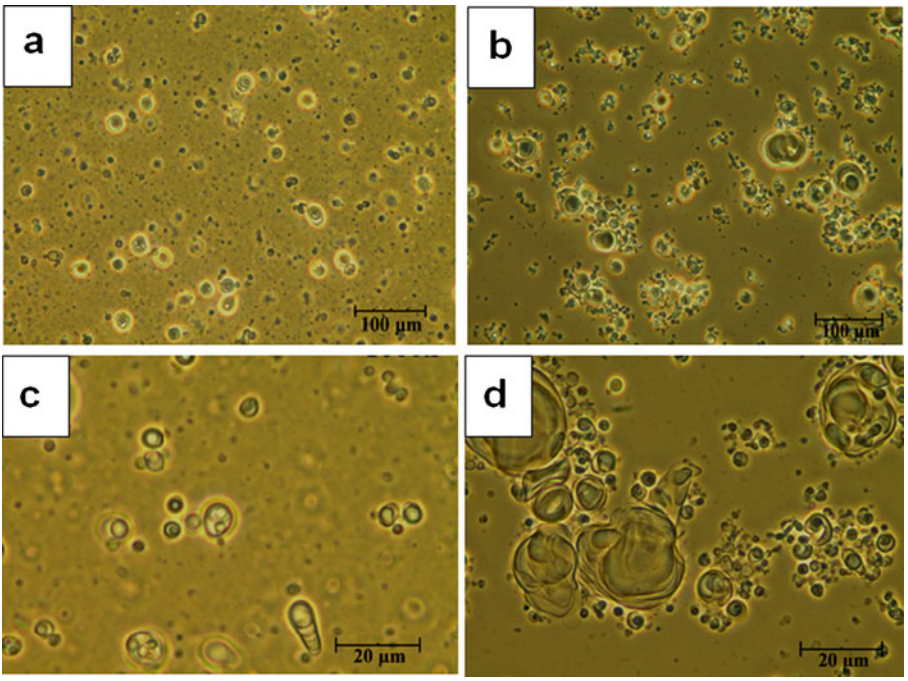

Fig. 3 Optical micrographs of **a** liposomes after three freeze–thaw cycles. **b** Liposomes after 15 min of agitation with 50 μl of Triton X-100. **c** Liposomes after three freeze–thaw cycles with higher magnification. **d** Liposomes after 15 min of agitation with 50 μl of Triton X-100 with higher magnification

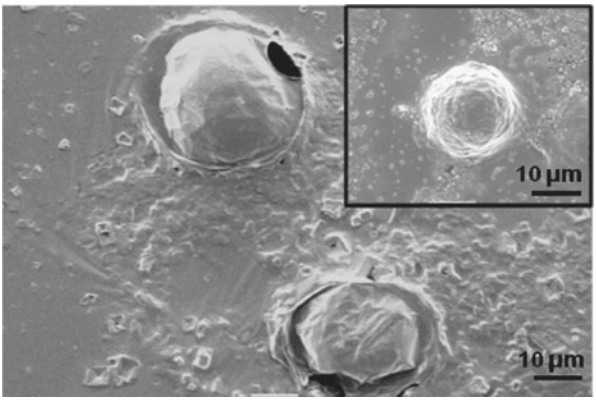

Fig. 4 SEM micrographs of liposomes after the freeze–thaw step (*inset*) and after membrane disruption with Triton X-100. The liposomes are 10 μm in size. Small PBS salt crystals are also visible in the images. The *dark areas* are openings in the membrane structures

much higher in the liposome than in solution. Therefore, if disruption occurs, ONPG should diffuse rapidly into the solution, yielding detectable release.

Control Experiments

The first control was to establish that the detergents in PBS do not contribute to absorbance at 420 nm. The absorbance of the detergent (Triton X-100) was found to be negligible (0.0036±0.0051 AU).

Another concern raised was that the liposome themselves may contribute to the absorbance readings in the release data. Lipids were found to have a significant absorbance at 420 nm. When a lipid cake was resuspended in 5 ml of 0.08 M PBS, pH 7.7, the absorbance at 420 nm was 2.0439 AU. This is most likely due to the turbidity rather than the specific absorbance of a wavelength, but it is still an issue.

Since not all liposomes formed are large enough to be centrifuged out of suspension, liposomes may still be in the supernatant and add to the absorbance at 420 nm, after being centrifuged another 20 min at $13.3 \times 1{,}000\ \text{min}^{-1}$ with detergents. The blank would also have these remnant liposomes, but it is necessary to test whether or not the disruptive nature of detergents causes liposome fragments to form or more non-centrifugable liposomes to be in the suspension.

To test whether or not detergents cause more lipids/liposomes to remain in solution during the release readings, the experiment was conducted without ONPG in the hydration solution. Therefore, any absorbance readings in the release tests would be from extra lipids/liposomes since the blank accounts for contributions from the buffer, β-gal, and the basal level of lipids/liposomes left in solution after centrifugation. Also, it was previously shown that the detergent's absorbance at 420 nm is negligible.

The lipid contribution to absorbance at 420 nm was found to be only 0.0587± 0.0325 AU. However, the absorbance due to the addition of 1.0% Triton X-100 was 0.1983 AU, which is significantly higher than 0.0587 AU, indicating release of ONPG.

It was found that Triton X-100 lowered the absorbance at 420 nm (Table 1). It is not yet determined if this is because of effects of Triton X-100 on the β-gal, ONPG, or ONP. Regardless of the reason, these effects must be taken into account. Therefore, a correction

Table 1 Effect of detergent on β-gal activity

	Absorbance at 420 nm (AU)	% Decrease in max absorbance due to detergent
No detergent	0.2457±.0145	N/A
1.0% Triton X-100	0.2203±0.0044	11.52±4.31%

factor was developed based on the percentage change in absorbance due to the presence of Triton X-100 detergent.

Liposome Release of ONPG

The detergent 1.0% Triton X-100 was used to lyse the liposomes. To determine the optimal detergent lysing duration, ONPG release was measured after varying amounts of time (Fig. 5). The most significant release occurs within the first 5 min. From 5 to 10 min, the increase is less, and from 10 to 20 min, statistically there is little increase at all. Release was quantified after 15 min of incubation because it has the statistically highest chance of being the time of maximum release after Triton X-100 is added. By subtracting the absorbance of solution with no detergent added (0.2167) from the post-15-min mean absorbance (0.4150), the resulting absorbance value of 0.1983±0.0626 AU was determined. This result was then corrected by using correction factors shown in Table 2.

Quantification of ONPG Encapsulated in the Liposomes

The moles of ONPG encapsulated in the liposomes were quantified by using a standard calibration curve for ONP absorbance (Fig. 6), which was developed by reading absorbance as a function of ONPG concentration. The standard curve produced a slope of 0.0039 nmol/AU, which can be used to convert absorbance at 420 nm to nanomoles of ONPG released, assuming a 1:1 stoichiometric relationship of ONPG to ONP conversion. Thus, the corrected reading of 0.1517 AU indicates that 31.68 nmol of ONPG was encapsulated in the liposomes, as shown in Table 3.

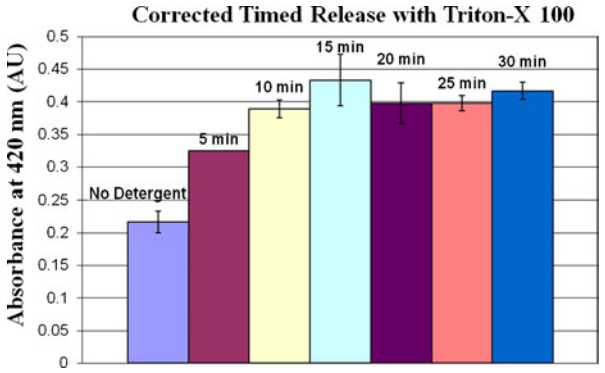

Fig. 5 The absorbance values of liposomal solutions after varying amounts of time spent incubating in Triton X-100. No detergent absorbance is .2167±.0168, 5 min absorbance is .325±.00009, 10 min absorbance is .3892±.0137, 15 min absorbance is .4334±.0396, 20 min absorbance is .3981±.0313, 25 min absorbance is .3982±.0117, and 30 min absorbance is .4169±.0132

Table 2 Application of correction factors

Sample	Absorbance at 420 nm
Raw release data (AU)	0.1983
Lipid contribution (AU)	−0.0587
Triton X-100 contribution (AU)	−0.0036
Detergent effect on β-gal	+11.52%
Corrected release data (AU)	0.1517

Corrected release data=(raw release data−lipid contribution−Triton X-100 contribution)+[(raw release data−lipid contribution−Triton X-100 contribution) 11.52%]

Discussion

Analyzing ONPG Release

This paper outlines the development of a method of detecting liposome release without having to deal with fluorescent dyes or nanoparticles which can skew data by shining through liposomes, photobleaching, or blinking. It also is much simpler than previous dyeless detection methods (mentioned in "Introduction" Section).

Chromophores/photophores incorporated within the liposomes cannot be used to quantify release since the liposomes are virtually transparent to their emissions; therefore, there will be no discernable differences between the optical signals from lysed liposomes and unlysed liposomes. The enzyme–substrate method described herein can also be a valuable control in determining the accuracy of release measurements and can help to verify the accuracy of chromo/photophore systems.

The total nanomoles of ONPG encapsulated divided by this number give the percent encapsulated, i.e., 6.33%, as shown in Table 3. The percent encapsulation is fairly low, but when the ratio of lipids to hydration solution is considered, it is understandable. To improve this number, either more lipids or less hydration solution should be used. If a hydration solution with a volume slightly higher than the encapsulated volume was used, it would eliminate lots of waste, as well as aid in the removal of liposomes from solution due to solubility issues.

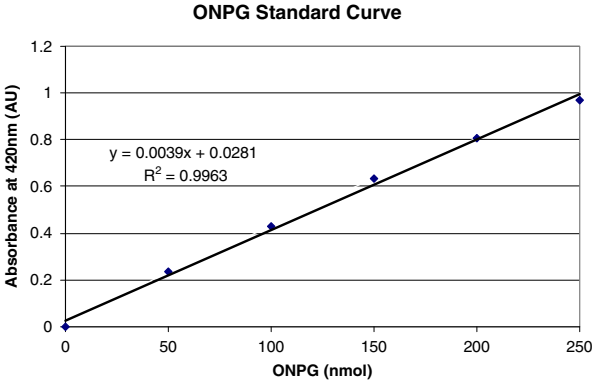

Fig. 6 The spectrophotometer was blanked with a solution of 800 μl 0.08 M PBS of pH 7.7, 100 μl 25 U/ml β-gal, and 50 μl of 1.0% Triton X-100. One hundred microliters of 2.5 mM ONPG in 20-μl aliquots was added, allowed ~2 min to react fully, and read at 420 nm

Table 3 Percent encapsulation

	Raw	Corrected
Absorbance at 420 nm (AU)	0.1983	0.1517
ONPG encapsulated per tube (nmol)	43.64	31.68
Total ONPG encapsulated (nmol)	218.20	158.42
Encapsulated hydration solution (μL)	436.4	316.8
Percent encapsulation	8.73%	6.33%

Future Work

The next phase of this research will address photoinduced lysis of liposomes. As mentioned previously, many fluorescent dyes have low photostability and can be photobleached by the activation of photoinducers. Therefore, this new release detection system will be perfect for testing photoinduced lysis since the lysing method (irradiation) will not affect the reporter system. We believe that this release method may be useful for in situ monitoring of liposome integrity in the presence of media (plasma, serum proteins, whole blood, etc.).

In future work, an ESEM can be utilized to visualize extruded liposomes [20]. This will provide a qualitative indication of the uniformity obtained and a better view of any disruptions which occur during lysis.

Furthermore, the percentage of detergent to use which gives the fastest and most complete membrane disruption will be investigated. This will speed up the procedure and improve percent encapsulation data.

Further optimization could be achieved by determining the best lipid to hydration buffer ratio to increase percent encapsulation. This way, valuable reagents are not wasted in the wash step, a particularly important consideration in the event of commercialization.

Currently, the majority of liposomes produced in this protocol are multilamellar liposomes (MLVs) of varying diameters greater than 500 nm. With the addition of an extrusion step, liposomes will be reduced to SUV, increasing the uniformity, and more importantly, the stability of the liposome encapsulates. Furthermore, when MLVs are disrupted with a detergent (Triton X-100), it is not certain how many layers were disrupted, and therefore, if all of the encapsulated substrate was released. In this paper, the unavailability of an ultracentrifuge limited us to working with MLVs.

Due to their small size, the liposomes created by extrusion will require an ultracentrifuge capable of 100,000×*g* for the centrifugation steps [18]. Extrusion will allow the completion of meaningful stability tests of ONPG encapsulation, as well as improve the precision of detection due to uniformity in solutions and completeness of lysis (as discussed above).

Although we were limited to MLVs in this research, it does not diminish the significance of the research. The liposome release yields reported here are lower than expected because of incomplete lysis, but the fact remains that the procedure we have developed is bona fide and has many advantages over a chromophore/photophore- based system.

Summary and Conclusions

A new approach for the detection of liposome lysis utilizing an enzyme–substrate system has been developed by using β-galactosidase (β-gal) and *o*-nitrophenyl-β,D-galactopyranoside (ONPG). After ONPG is encapsulated inside 1,2-dimyristoyl-*sn*-glycero-3-phospho-

choline (DMPC) liposomes, 1% Triton X-100 detergent is used to lyse the liposomes and release the ONPG. The cleavage of the ONPG by β-gal produces *o*-nitrophenol (ONP), whose optical absorbance is at 420 nm, provides an indication of the quantity of ONPG release from the liposomes. The experimental results indicated that the most significant release from detergent-lysed multilamellar vesicle liposomes occurs within the first 5 min, while the release process is 99% complete after 15 min.

Consideration of separate contributions of the detergent, the lipids, and the effect of detergent on β-gal activity to the total absorbance allowed us to develop correction factors, which can be applied to the release data. The percent encapsulation can be determined from a standard curve that expresses absorbance as a function of nanomoles of ONPG, which can be corrected using these factors.

We have shown that the enzyme–substrate system is a promising tool for detection of liposome lysis due to its simplicity and utility where chromophores/photophores cannot be used. In the broader context, the system could also be utilized in the in situ monitoring of liposome stability in cell culture.

Acknowledgments This work was all done at ERDC-CERL and was funded by USACE 6.1 funds. The authors would also like to thank Ms. K. L. Whalen for aid in editing the paper.

References

1. Bangham, A. D., Standish, M. M., & Watkins, J. L. (1965). *Journal of Molecular Biology, 13*(1), 238–252.
2. Ozato, K., Ziegler, H. K., & Henney, C. S. (1978). *Journal of Immunology, 121*(4), 1376–1382.
3. Sessa, G., & Weissmann, G. (1970). *The Journal of Biological Chemistry, 245*(13), 3295–3301.
4. Gregoriadis, G. (Ed.). (1984). *Liposome Technology* (Vol. 1). Boca Raton: CRC Press.
5. Gomes, J., & Baptista, A. (2001). *Textile Research Journal, 71*(2), 153.
6. Chaize, B., Colletier, J., Winterhalter, M., & Fournier, D. (2004). *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology, 32*(1), 67–75.
7. Schreier, H., & Bouwstra, J. (1994). *Journal of Controlled Release, 30*(1), 1–5.
8. Bangham, A. D., & Home, R. W. (1964). *Journal of Molecular Biology, 8*, 660–668.
9. Chen, C., Yao, J., & Durst, R. (2006). *Journal of Nanoparticle Research, 8*(6), 1033–1038.
10. Hatzi, P., Mourtas, S., Klepetsanis, P., & Antimisiaris, S. (2007). *International Journal of Pharmaceutics, 333*(1–2), 176.
11. Mourtas, S., Duraj, S., Fotopoulou, S., & Antimisiaris, S. (2008). *Colloids and Surfaces. B: Biointerfaces, 61*(2), 270–276.
12. Peschka, R., Dennehy, C., & Szoka, F. C. (1998). *Journal of Controlled Release, 56*(1–3), 41–51.
13. Resch-Genger, U., Grabolle, M., Cavaliere-Jaricot, S., Nitschke, R., & Nann, T. (2008). *Nature Methods, 5*(9), 763–775.
14. Larson, D. R., Heikal, A., Ow, H., Srivastava, M., Wiesner, U., Baird, B., et al. (2003). *Biophysical Journal, 84*, 586A.
15. Switzer, R., & Garrity, L. (1999). *Experimental biochemistry: Theory and excercises in fundamental methods* (pp. 123–134). New York: W.H. Freeman and Company.
16. Weissman, G., Sessa, G., & Weisman, S. (1965). *Nature, 208*, 649–655.
17. Kinsky, S. C., Haxby, J., Kinsky, C. B., Demel, R. A., & VanDeenen, L. L. M. (1968). *Biochimica et Biophysica Acta, 152*(1), 174–185.
18. Glick, D. (1988). *Methods of Biochemical Analysis* (Vol. 33, pp. 337–462). Hoboken: Wiley.
19. Anderson, S., & Krinsky, N. (1973). *Photochemistry and Photobiology, 18*(5), 403–408.
20. Mohammed, A. R., Weston, N., Coombes, A. G., Fitzgerald, M., & Perrie, Y. (2004). *International Journal of Pharmaceutics, 285*(1–2), 23–34.