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13. ABSTRACT (Maximum 200 Words) This study investigated the assembly of a biomimetic sensor containing an osmotic receptor protein. The first objective of this research included the assembly and immobilization of fluorescently tagged liposomes. The second objective involved the expression and purification of an osmotic sensitive protein (MscL) and the incorporation of this protein into the liposome membrane. Liposomes (2 micron diameter) containing fluorescein labeled phospholipids and biotinylated phosphatidyl ethanolamine in the membranes and internalized soluble sulforhodamine were assembled. Liposomes were characterized with respect to composition, size, and shelf-life using confocal microscopy. Avidin was covalently attached to a glass surface for the immobilization of the biotinylated liposomes. Immobilization of fluorescent liposomes was confirmed with confocal microscopy. The liposomes contain a green/yellow lipid bilayer and a red interior. The cloning of recombinant MScL into an <i>Escherichia coli</i> expression system yielded an MscL-thioredoxin fusion protein that was tagged with a blue fluorescent dye and incorporated into the membrane of the liposomes. The functionality of the proteosome was observed by the release of the water soluble sulforhodamine in the presence of high salt, 3 M, concentrations.			
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Biomimetic Sensor

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Project Summary

The objective of this work is the incorporation of a known osmotic receptor protein into a liposome or pseudo-cell for the long term goal of producing a chemo/thermo sensitive biomimetic. Initially the composition, size, immobilization and stability of liposomes will be addressed. The second phase involves the cloning, expression and purification of the MscL protein from *E. coli* and the incorporation of this protein into the membrane of the liposomes.

The technical objectives of this research are:

1. To characterize fluorescently tagged immobilized liposomes with respect to composition, size, stability and density. Visualization of liposomes will be done by confocal microscopy.
2. To incorporate the recombinantly produced MscL receptor into the membrane of the liposomes.

Materials and Methods

Liposome composition

The liposome components, phosphatidylcholine, phosphatidylglycerol, fluorescent NBD-phosphatidyl ethanolamine, cholesterol, fluorescein labeled phosphatidyl choline, sulforhodamine B, and biotinylated phosphatidyl ethanolamine were varied one at a time to produce soluble liposomes. The fluorescent lipids were purchased from Molecular Probes (Eugene, OR) and the other components were purchased from Sigma Chemical Co. (St. Louis, MO). Initially, sulforhodamine B was not used to determine the optimum ratio of the labeled to unlabeled phospholipids. All liposome preparations were cast in 50 mM Tris, pH 7.2.

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Sample preparation and stability assay

The homogenizer used in all experiments was a Microfluidizer 110S with a 10 ml sample hold volume, capable of 10,000 psi and an optional cooling loop. The number of passes through the homogenizer vs. liposome size showed that homogenization for 5 minutes (samples tested from 1 to 12 minutes) was sufficient for the production of 2 μ m diameter liposomes. The shearing of the piston pump generates heat, which is transferred to the solution during homogenization. Increasing the homogenization time greater than 5 minutes would require the use of the cooling coil. Other sample preparation conditions examined included the use of a filter and centrifugation to concentrate the sample. These conditions resulted in aggregation of formed liposomes, therefore we are not using these sample preparation techniques.

Immobilization and stability of liposomes

Glass slides were derivitized with 3-aminopropyl-triethoxysilane followed by succinylation with succinic anhydride and acetylation with acetic anhydride. Avidin (0.6 ng avidin per 18 x 18 nm coverslip) was covalently immobilized to the derivitized slides via EDC and sulfo-NHS (Pierce Chemical Co.). Biotinylated liposomes prepared as described above were incubated with the avidin-slides, followed by washing the slides with PBST containing 1M NaCl to remove nonspecifically adsorbed liposomes.

The stability of immobilized liposomes was determined by storing the samples dry at 4°C for four months. The samples were imaged every two weeks during this time period.

PCR Protocols

The two primers used in PCR were designed from published *E. coli* MscL sequences namely EmsclFor and EmsclRev. The primer sequences are ATGAGCATTATTAAAGAATTTTCG and CCAGTGGCAAGAAAGTAAATC for EmsclFor and EmsclRev, respectively. A PCR kit (Read-To-Go PCR, Amersham Pharmacia Biotech, Piscataway, NJ) was used for all PCR experiments. The PCR sample consisted of 1 μ M of each primer, 1nM of *E. coli* genomic DNA as the template, 1 μ l of Tac polymerase, and 22 μ l of sterile water. The cycling conditions for PCR included: 94°C/1 min, 52°C/1 min, and 72°C/1 min with a final 10 minute extension at 72°C.

Cloning and sequencing of MscL

The cloning vector and host used in this experiment were purchased from Invitrogen (Carlsbad, CA). The vector, pBAD/Thio, includes an ampicillin resistance gene and the cloning site is surrounded by HP-thioredoxin, and 6xHis tag at the target protein N-terminal and C-terminal. The *E. coli* host cell, TOP10 has a genotype of $F^- mcrA \Delta(mrr-hsdRMS-mcrBC) \phi80lacZ\Delta M15 \Delta lacX74 recA1 deoR araD139 \Delta(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG$.

For cloning into the pBAD vector, the cloning reaction included 2 μ l PCR product, 2 μ l sterile water, 1 μ l salt solution, and 1 μ l TOPO vector (as supplied by Invitrogen). After a 1 hr incubation at room temperature the cloning reaction was transformed into competent TOP10 and plated onto agar plates containing ampicillin. Ten colonies were picked the following day and assayed for plasmid DNA size. Colonies containing the correct insert size were purified and sequenced. The forward and reverse primers for sequencing were supplied by Invitrogen and the sequencing was performed at the USU Biotechnology Center (Logan, UT).

E. coli cell growth and MscL induction

Recombinant *E. coli* was grown in 2 ml LB containing 100 μ g/ml ampicillin overnight at 37°C with shaking (225 rpm). The overnight growth was subcultured into five tubes each containing 10 ml of LB with 100 μ g/ml ampicillin. Samples were grown at 37°C with shaking (250 rpm) to mid-log phase ($OD_{600} = 0.5$). A 1 ml aliquot of each sample was removed, pelleted and stored at -80 C as the zero induction point.

For induction of the MscL-thioredoxin fusion protein, arabinose was added to each of the five tubes at final concentrations of 0.00002%, 0.0002%, 0.002%, 0.02% and 0.2%. Samples were grown at 37°C with shaking for 4 hours. Aliquots, 1 ml, from each tube were collected and the cells pelleted. The cell pellets were resuspended in 100 μ l of 1X SDS-PAGE sample buffer along with zero time point samples collected as described above. Samples were heated for 5 minutes at 70°C and 10 μ l of each sample analyzed on a 10% SDS-PAGE gel.

Protein purification

Recombinant cells were grown in 500 mL of LB/Amp to mid log phase. Cells were pelleted and resuspended in 5 ml B-PER reagent (Pierce, Rockford, IL) containing 0.65 μ l of 0.1

M PMSF. The sample was centrifuged and after careful removal of the supernatant 5 ml of B-PER reagent was added and the pellet was resuspended. Lysozyme (100 μ l of 10 mg/ml stock solution) was added to the suspension for a final concentration of 200 μ g/ml. After incubation for 5 min at room temperature, the soluble fraction was separated from the insoluble fraction by centrifugation at 10,000 rpm for 20 minutes.

The ThioBond resin, 2 ml as supplied by Invitrogen, was equilibrated according to the manufacturers directions and the lysate was applied and incubated for 30 min at room temperature. The resin was drained and washed with 4 bed volumes (8 ml) of wash buffer containing 1 mM β -ME. The fusion protein was eluted with 3 bed volumes (6 ml) of running buffer with increasing β -ME concentrations of 5, 10, 50, 100, 200, and 500 mM β -ME. Elutants were collected and analyzed by SDS-PAGE.

Fluorescent labeling of recombinant MscL

Recombinant MscL-thioredoxin was labeled with 5-(and-6)-carboxynaphthofluorescein (CNF) which has an emission at 668 nm and an absorbance maximum at 598 nm and appears as a blue color. Purified recombinant MscL-thioredoxin (0.8 μ M) was reacted with EDC (4.8 μ M) and Sulfo-NHS (13 μ M) followed by 1.5 μ M of CNF. The labeled protein was dialyzed overnight against 50 mM Tris to removed excess CNF.

Liposome formulations containing MscL

Liposomes containing the lipids described previously were formed in the presence of 0.2 nM thioredoxin-MscL-CNF. Liposomes were formed at 10,000 psi for 4 min followed by immobilized onto avidin slides as described above.

Functionality of liposomes containing MscL were visualized using confocal microscopy. Each slide containing immobilized liposomes was placed on a petri dish containing an 18 x 18 hole. Liposomes were imaged in the presence of 50 mM Tris, pH 7.2 followed by a salt wash at approximately 3 M NaCl.

Result

Liposome composition and stability

The final composition of the liposomes used in this study is shown in **Table 1**. At the concentrations used, the lipid bi-layer and the interior containing the soluble sulforhodamine B are visible. **Figure 1** is a confocal picture (60 x) of the soluble liposomes without the incorporation of sulforhodamine B. These liposomes are of uniform 2 μ diameter. The lipid bilayer in the liposomes is shown clearly in **Figures 2 and 3**. Figure 3 is a magnification of the liposomes and incorporates sulforhodamine B in the interior. In this case, the color of the liposomes changes from the green initial color to a yellow color with the incorporation of sulforhodamine B.

Table 1. Liposome composition

Component	Final Concentration
phosphatidylcholine,	300 micro M
phosphatidylglycerol	40 micro M
NBD-phosphatidyl ethanolamine	0.6 micro M
cholesterol,	60 micro M
fluorescein labeled phosphatidyl choline	0.02 micro M
sulforhodamine B	1.8 micro M
biotinylated phosphatidyl ethanolamine	0.6 micro M

Immobilized liposomes were visualized with confocal microscopy (**Figure 4**). Figure 4 shows the immobilized liposomes at 100 x magnification. There are both yellow and red liposomes which is due to the different concentrations of sulforhodamine B in the interior. We have tested the stability of the immobilized liposomes stored at 4°C for 3 months, and there was no decrease in the number of liposomes per field. After 3 months approximately 50% of the liposomes had destabilized which was evident by the absence of structure and the precipitated sulforhodamine B (**Figure 5**).

Figure 6 is a Z series scan of the immobilized liposomes. The exterior of the liposomes is green/yellow in color while the interior is red/yellow. This demonstrates that different dyes were incorporated into uniform liposomes that were then immobilized onto a solid support.

PCR and cloning experiment

The PCR primers designed are from published *E. coli* MscL gene sequence. The forward primer EMSCLFor begins at the start codon (ATG) at the 5' end of MscL to ensure the correct reading frame and translation process. The reverse primer EMSCLRev includes a stop codon and terminator sequence to ensure sequence termination.

Two different annealing temperatures were used to determine suitable PCR conditions. An annealing temperature of 52°C compared to 50°C generated more specific PCR product at the expected 481 base pair product size (**Figure 7**). Therefore, this temperature was used in following PCR experiments.

The PCR product was cloned into the TOPO vector and two positive clones were identified, MscL2 and MscL6 (**Figure 8**). Although MscL6 contained a PCR insert, sequencing confirmed the presence of multiple errors in the base-pair sequence. MscL2 contained one base-pair error, which changed one amino acid in the target MscL protein sequence from valine to alanine (**Figure 9**). Since these two amino acids share similar chemical properties, we assumed this change would not affect MscL protein functionality.

Gene induction and MscL protein purification

The pBAD/TOPO vector was selected because the TOPO vector is easier and faster than traditional PCR cloning vectors and this vector contains the araBAD promoter, which is tightly controlled by the arabinose concentration in medium. Previous expression of MscL gene using the T7 promoter was unsuccessful, therefore, it is assumed that high level expression of this protein is lethal to the host cell. Use of the tightly controlled arabinose promoter ensures that this lethal effect will not occur during the cell growth phase. In addition, the fusion protein containing a thioredoxin tag tends to be more soluble in cell plasma than other affinity tags allowing the affinity purification using a nickel column. The thioredoxin tag is linked to the N-terminal of target protein via an EK site, which can be used for EK proteinase cleavage to remove the thioredoxin tag. The calculated molecular weight of the MscL fusion protein 28 kDa.

The expression of the MscL fusion protein was maximum at 0.002% arabinose in the medium (**Figure 10**). Higher arabinose concentrations resulted in less protein expressed which may be due to the lethality of this protein to the host at the high expression rate.

The initial attempt to lyse the induced cells with B-PER failed to release recombinant MscL protein into the soluble lysate. Since MscL is a membrane protein, we added an additional lysozyme treatment after treating with B-PER to degrade the cell wall. As expected, recombinant MscL was released into supernatant (**Figure 11**). Use of the ThioBond resin for affinity purification did not result in purified recombinant fusion protein. The recombinant protein did bind to the resin, but could not be released with any of the concentrations of β -ME tested (**Figure 12**). To confirm the fusion protein had bound to the affinity resin, the resin was boiled in SDS-PAGE loading buffer. The released protein was analyzed by SDS-PAGE and showed a protein band at 28 KD, but this purified protein could not be used in the liposome experiments. We concluded that the almost irreversible binding of the recombinant protein to the resin was due to the specific binding properties of MscL, not the thioredoxin.

Since the recombinant fusion could not be purified using the nickel resin the protein released after lysozyme treatment was used in further experiments (**Figure 11**, pellet after step 2). Scanning densitometry of this released protein shows that 40% of the total protein is 28 kDa, which we assumed to be the MscL-thioredoxin fusion protein. This sample was labeled with CNF with a 1.8:1 CNF:protein ratio and incorporated into liposomes. **Figure 13** shows the liposomes which were previously yellow/green to appear blue with the incorporation of the labeled protein.

The functionality of the incorporated MscL was tested using a change in ionic strength. The MscL membrane protein forms a large pore in the presence of high salt concentrations. The immobilized liposomes contained internalized, water soluble sulforhodamine B which should be released upon the opening of the pore. Although the orientation of the membrane protein can not be determined, we found that with the addition of high salt to the MscL-liposomes, the internalized sulforhodamine B was released, yet the liposome integrity is unchanged (**Figure 14**). We concluded that the sulforhodamine left the liposomes via the membrane pore in the presence of high salt. Since the release of the internalized sulforhodamine B is instantaneous in the presence of high salt concentrations, the visualization of these liposomes was done using the red laser. Therefore the liposome image is difficult to see in **Figure 14 a**, but is apparent in **14 B**.

Controls included liposomes which did not contain MscL in the membrane. With these samples, the sulforhodamine remained in the interior of the liposomes.

Conclusions

We have developed an immobilized liposome system that is stable for at least 3 months stored dry at refrigeration temperatures. The composition of the liposomes allows imaging using confocal microscopy. The combination of the green lipid bilayer with the red soluble dye interior is novel. The addition of a blue-labeled membrane protein, MscL, into the system was also visualized with confocal microscopy. The functionality of the membrane protein was demonstrated with a change in the osmotic conditions. With an increase in ionic strength, the interior water soluble dye was released without losing the integrity of the liposome.

In summery, immobilized liposomes containing a yellow/green bilayer, a red interior and the blue MscL protein were created. The osmotic sensitivity of the protein was monitored by the release of the red interior dye from the liposome.

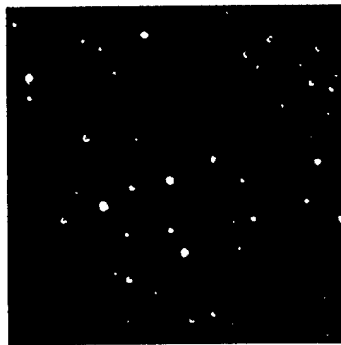


Figure 1. Soluble liposomes. Initial formulation without Sulforhodamine B. Note green color.

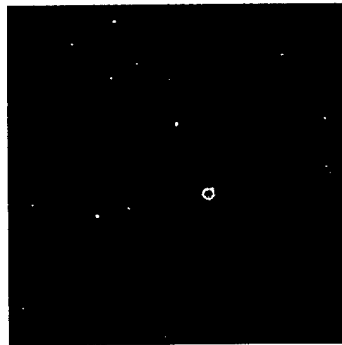


Figure 2. Soluble liposomes. Note lipid bilayer and green color.

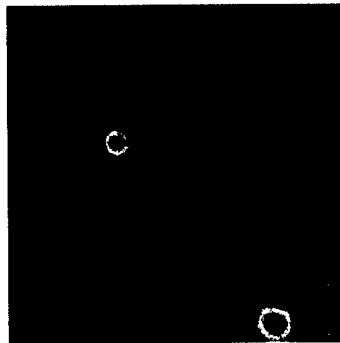


Figure 3. Soluble liposomes. Magnification of liposomes showing bilayer with Sulforhodamine in the interior. Note yellow color.

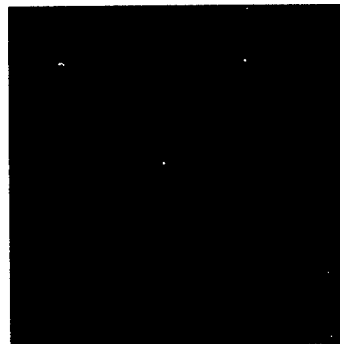


Figure 4. Immobilized liposomes at 100 x. Note the presence of both red and yellow liposomes.

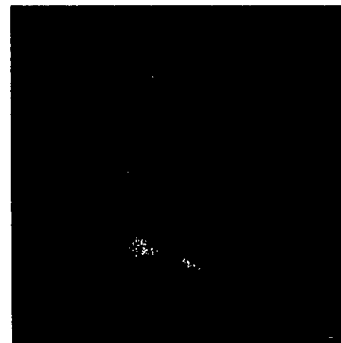


Figure 5. Immobilized liposomes imaged 4 months after immobilization. The lysozome has destabilized, releasing the internalized sulforhodamine B.

A



B



C

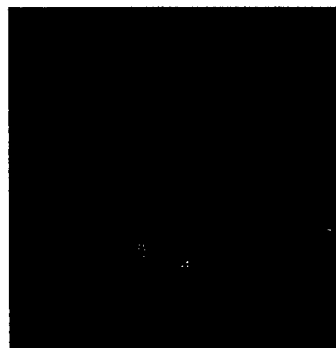


Figure 6. Immobilized Z series liposomes. Three cuts showing back (A), middle (B), and front (C). Note green exterior and red interior.

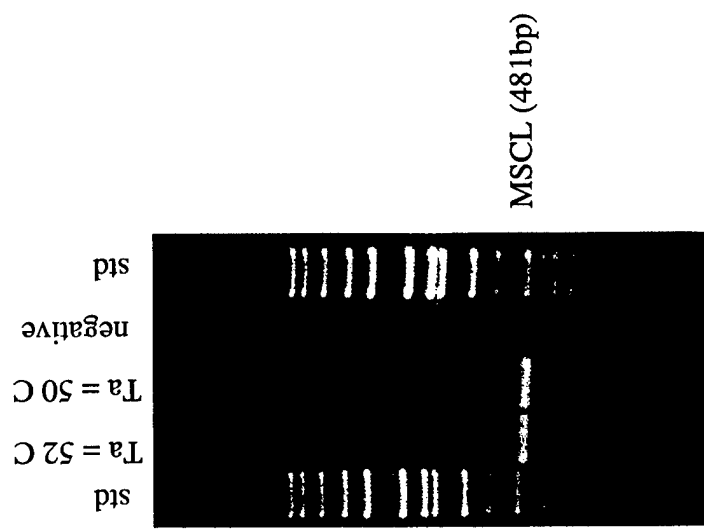


Figure 7. PCR amplification of the MSCL gene from E. coli

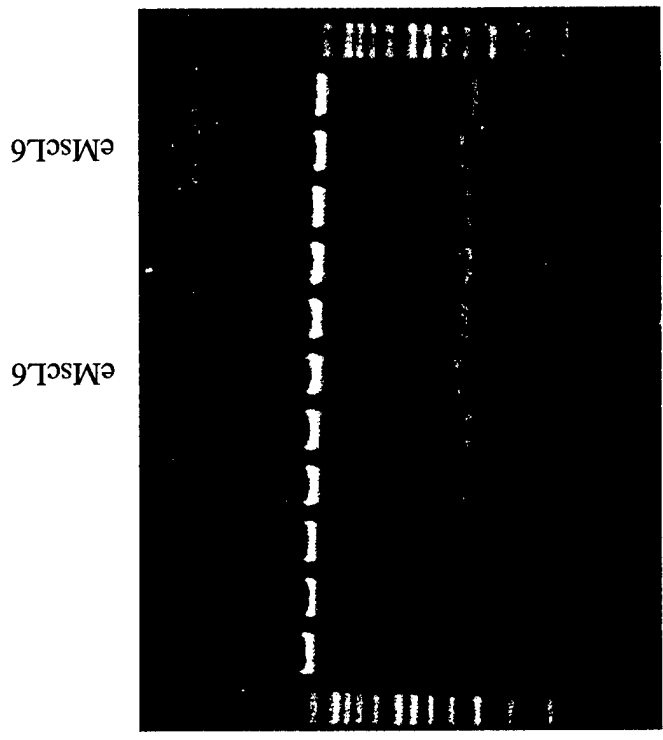


Figure 8. MscL PCR product cloned into pBAD/ThioFusion vector

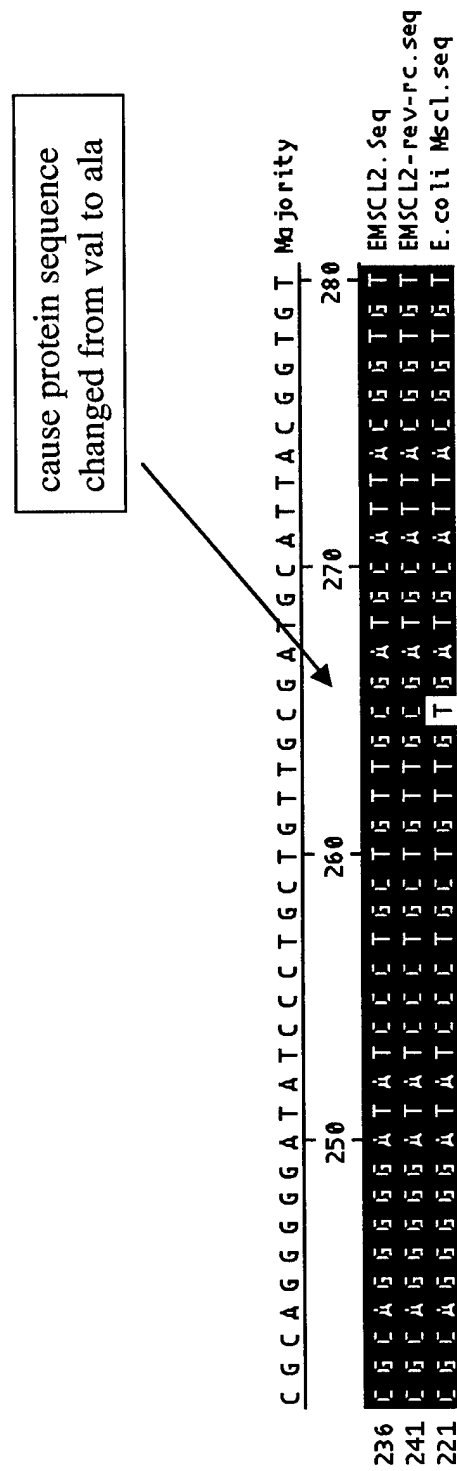


Figure 9. Sequence Alignment of recombinant MSCL with published *E. coli* MscL.

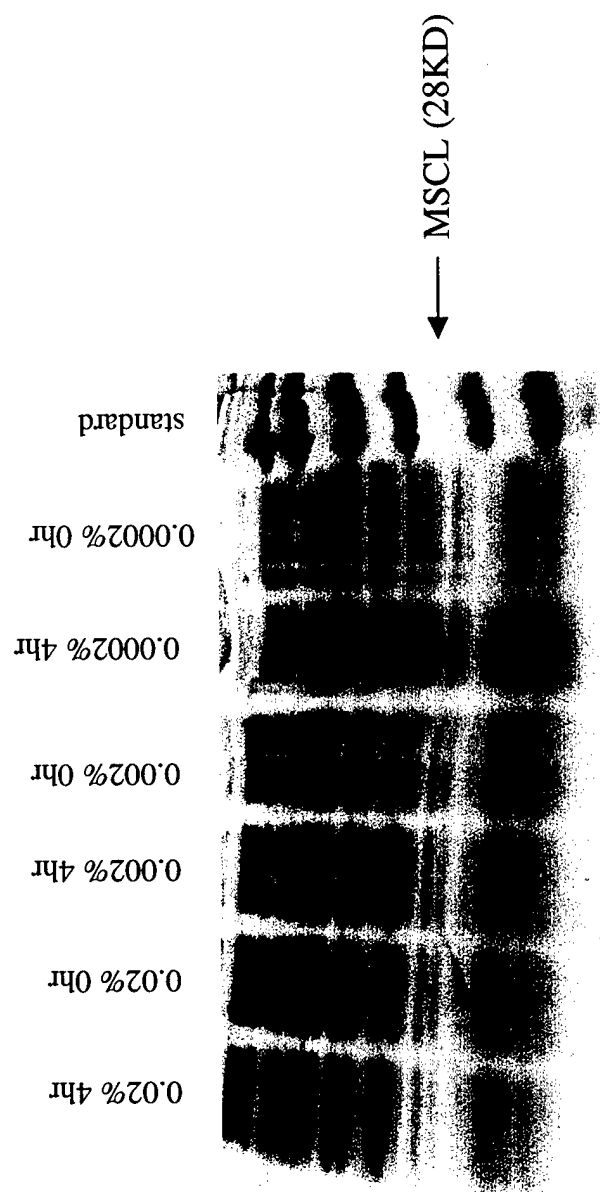


Figure 10. Induction of MSCL by different concentrations of arabinose

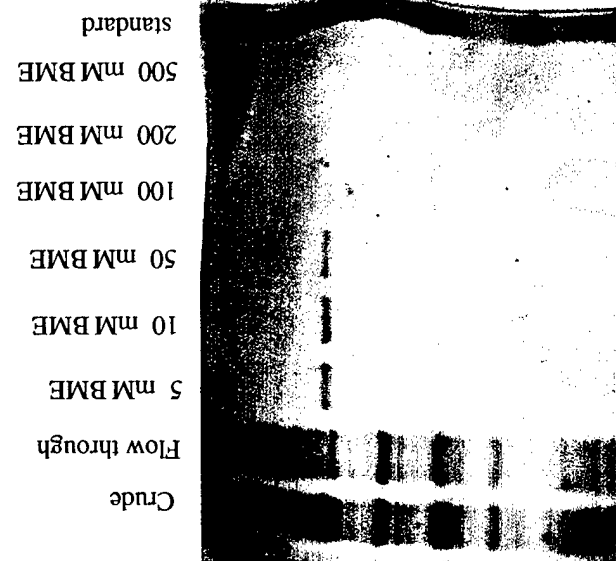


Figure 12. Elution of MscL from ThioBond resin.

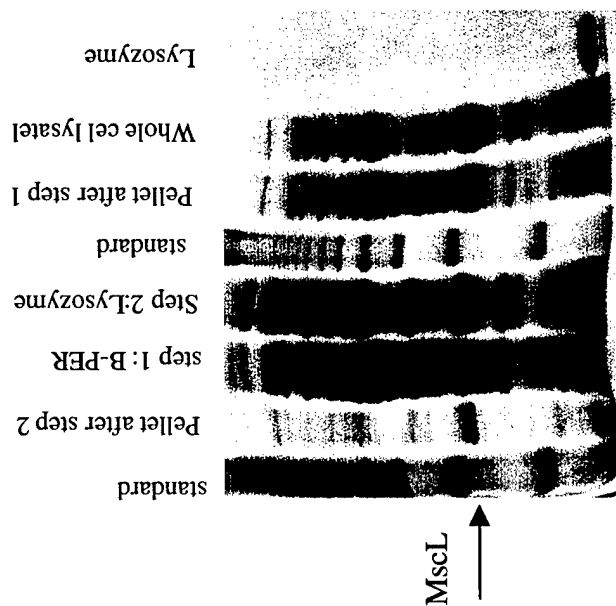


Figure 11. Localization of MscL in host cell by SDS-Page analysis.

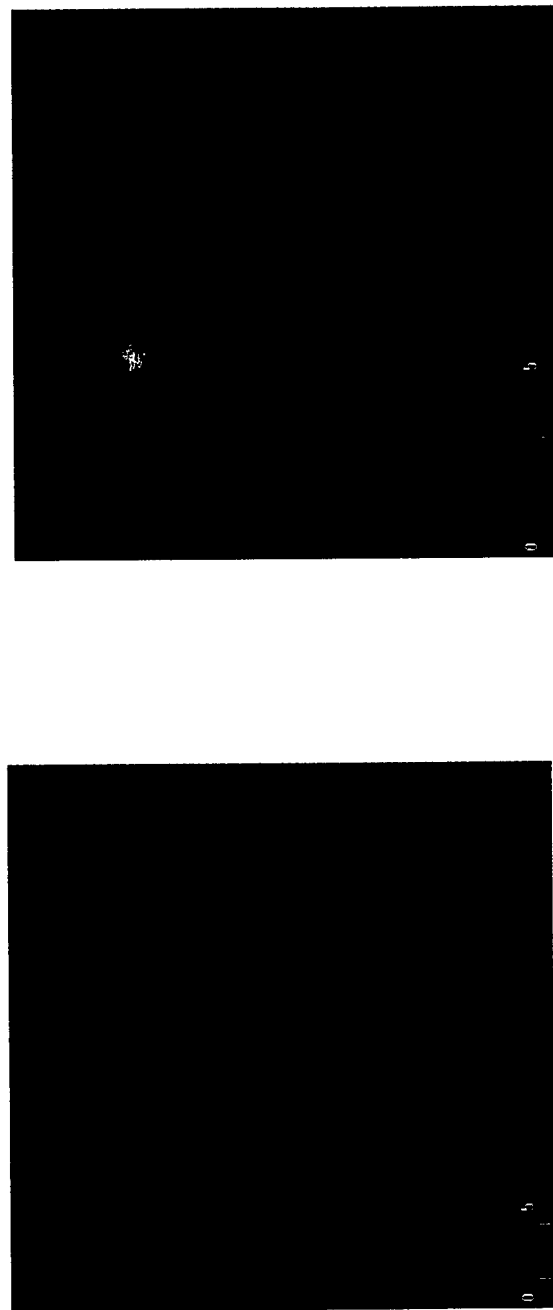


Figure 13. Confocal images of liposomes containing CNF-labeled recombinant MscL.

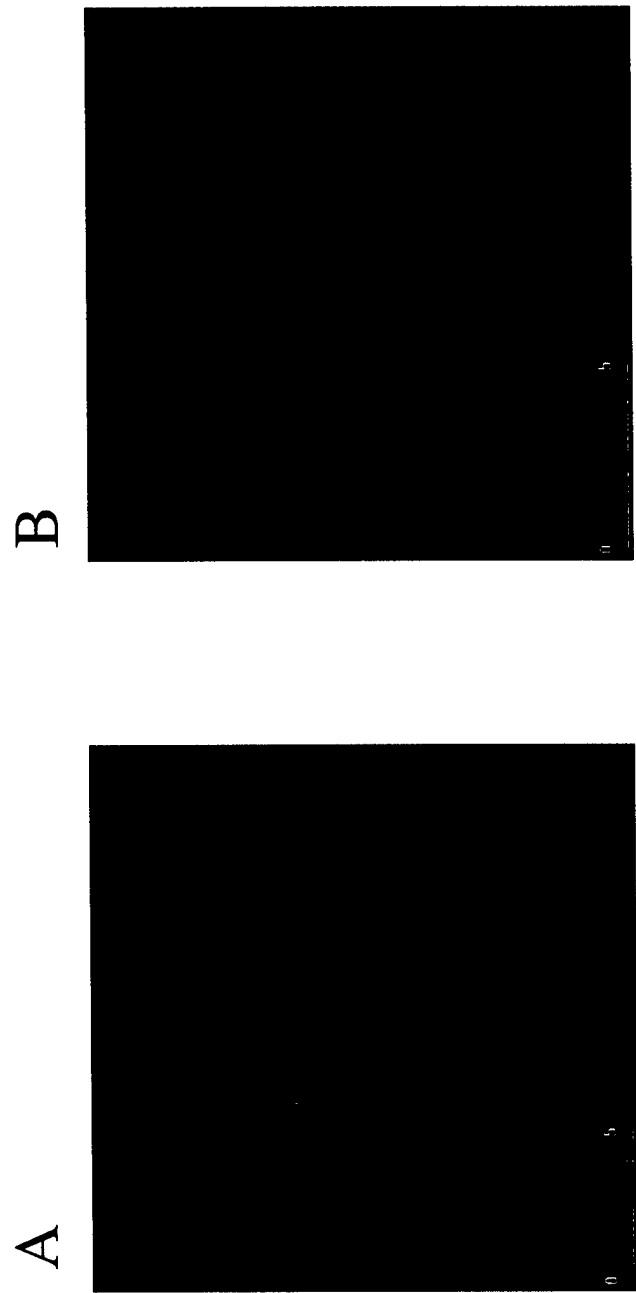


Figure 14. Confocal images of liposomes containing CNF-labeled before (A) and after salt treatment (B).