Photopolymerization of Polydiacetylene in Hybrid Liposomes: Effect of Polymerization on Stability and Response to Pathogenic Bacterial Toxins

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ABSTRACT

Liposomes containing lipids and polydiacetylene (PDA) are hybrid systems encompassing both a fluid phospholipid membrane and a polymer scaffold (PDA). However, the biophysical role of PDA in such liposomes is not well understood. In this report, we studied the effects of photo-polymerization of PDA on the stability of lipid-PDA liposomes, and their sensitivity to selected purified toxins and bacterial supernatants, using a fluorescence assay. Of the three different types of liposomes with variable lipid chain lengths that were chosen, the degree of polymerization had a significant impact on the long-term stability, and response, to external microbial exotoxins secreted by pathogenic bacteria, namely *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The degree of polymerization of

TCDA played an important role in lipid-chain-length-dependent stabilization of lipid-PDA liposomes, as well as in their response to bacterial toxins of *S. aureus* and *P. aeruginosa*.

KEYWORDS

Lipid-PDA liposomes, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, bacterial toxins, rhamnolipid, delta toxin

1. INTRODUCTION

Polydiacetylene (PDA) belongs to a family of π-conjugated polymers which display a unique colour change in response to various external stimuli such as: changes in pH;[1,2] temperature;[3] addition of chemicals;[4,14] mechanical stresses;[5] and biological substances[13,15] including bacteria and viruses.[6,7,8] The rapid colorimetric response allows PDA liposomes to be used for microbial detection based on expression of proteins by certain bacteria.[9,10] The amphiphilic nature of PDA molecules allows them to self-assemble within a monolayer, a planar bilayer or stable liposomes.[12] Blue to red colour transition occurs when an external stimulus disturbs the head group of PDA, resulting in a stress-induced conformational change in the polymerized backbone of the PDA bilayer.[12]

Despite the promising outlook of PDA liposomes in sensor application, the indiscriminate response to a variety of external stimuli limits practical application. The lipids in PDA liposomes are linked *via* laterally cross-linked polymer chains, thereby restricting the fluid nature of the membrane. It is desirable to synthesize the liposomes with membrane fluidity, which is an essential cell membrane characteristic, for functional interaction with extracellular proteins and molecules.[11,12] A substantial proportion of PDA is required for an intense colour change to take place, thus limiting the lipid content, resulting in poor bilayer membrane fluidity.[6,7,17,18] Alternative PDA liposomes – incorporating intercalated biological

or synthetic lipids - can mimic the fluid nature of cell membranes.[16,17,18,19] However the inclusion of free (non-polymerized) lipids in PDA liposomes decreases the colourimetric response.[18,19]

This study formulated lipid-PDA liposomes, which contain fluid lipid domains that can interact with bacterial toxins, spatially distinct from the polymerized domains of PDA. Our approach addressed two central questions regarding the role of phospholipids in PDA liposomes; how the phospholipids influence the polymerization of PDA monomers, and to what extent this affects the bilayer fluidity and the structural stability of lipid-PDA liposomes. We studied the effect of PDA polymerization on liposome stability *via* the progressive measurement of passive fluorescent dye release, and the liposome sensitivity to cytolytic toxins and supernatants of two selected strains of pathogenic bacteria, namely *Staphylococcus aureus* LAC and *Pseudomonas aeruginosa* PAO1 strains. We found that a low temperature annealing time of the liposomes, prior to UV activation, played an important role in the effective lipid-chain-length-dependent PDA polymerization, and consequently in the overall stability and sensitivity of polymerized lipid-PDA liposomes to bacterial cytolytic toxins.

2. EXPERIMENTAL SECTION

2.1. Materials: All materials used in the synthesis of lipid-PDA liposomes were commercially supplied and utilised without further purification. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol were obtained from Avanti Polar Lipids Inc (USA). 10,12-Tricosadiynoic acid (TCDA), 5,6-carboxyfluorescein (CF) and non-ionic detergent Triton X-100 were purchased from Sigma Aldrich (UK). All salts and

reagents used in preparing HEPES buffer were of molecular grade, and the solvents used were of analytical grade. Bacterial culture media, Luria Broth (LB) and Tryptic Soy Broth (TSB) were supplied by Fisher Scientific (UK) and Fluka (UK) respectively. Purified biosurfactant rhamnolipid (R-95 rhamnolipid) from *P. aeruginosa* was purchased from Sigma Aldrich (UK). The delta toxin (delta hemolysin), was synthesized by Biomatik, based on the sequence provided in the reference.[26]

2.2. Liposome preparation: Lipids, cholesterol and TCDA were individually prepared in chloroform to a standard concentration of 100 mmol dm^{-3}. TCDA in chloroform required ultra-sonication in water bath at 40 °C for complete dissolution. Lipids, cholesterol and TCDA were subsequently combined in the ratio of 55: 20: 25 (% V/V) respectively, as 25 % TCDA has been shown to exhibit optimum liposome stability and sensitivity.[20] 300 µl of the lipid mixture was evenly dried under nitrogen prior to placement in a vacuum chamber at 10^{-3} mbar for an hour to remove the chloroform. 50 mmol dm^{-3} of 5,6-carboxyfluorescein was prepared in HEPES (adjusted to pH 7.3) and it was self-quenched (Figure S3 in Supporting Information). No significant pH quenching of 5,6-carboxyfluorescein was observed between pH 4 and 10 (Figure S4 in Supporting Information). The dried lipid film was then rehydrated in 5 ml of 50 mmol dm^{-3} 5,6-carboxyfluorescein and heated in a water bath for 10 minutes at 70 °C, before being subjected to three freeze-thaw cycles. The resulting lipid-carboxyfluorescein solution was then extruded three times through a polycarbonate membrane of 100 nm diameter pore size using an LF-50 LiposoFast extruder (Avestin, USA). Finally, non-encapsulated carboxyfluorescein was removed by means of passing the solution through a DNA grade Illustra NAP-25 column (GE Healthcare, UK). Following the liposome preparation procedure above, control liposomes in HEPES (pH 7.3) were prepared for the UV-Vis absorbance control measurements.

2.3. UV polymerization of liposomes: Control- and carboxyfluorescein-containing liposomes were placed in quartz vial and polymerized under UV-light (254 nm) with a total dose of 90 mW cm^{-2} using a commercial flood exposure UV source (Hamamatsu, Japan). Uniform UV exposure was achieved by recirculating the liposomes during 3 separate exposures of 30 mW cm^{-2}. For each liposome type, eight different UV polymerizations were carried out, namely: no polymerization, polymerization directly after liposome extrusion and column purification, and polymerization after each day of storage in fridge at 4 °C for up to six consecutive days. All liposomes were then stored at 4 °C until use.

2.4. Fluorescent assay: According to the solubility diffusion model, the permeability of a lipid bilayer membrane with respect to water and water soluble small molecules is directly related to the fluid nature of the lipids in the bilayer.[21] This is characterized by measuring the degree of passive leakage of encapsulated fluorescent molecules, over a period of 2 hours in the desired medium, at 37 °C. Fluorescent measurements were carried out on a SPECTROstar Omega micro-plate reader (BMG Labtech) with a 96 well plate, using excitation and emission wavelengths of 485 ± 12 and 520 nm respectively, with a gain of 600. All experiments, with the exception of the 7 day long-term stability test, were carried out on 20 µl of vesicles inoculated with 60 µl of buffer/ supernatant/toxins in each well. In order to yield a standard deviation value, each vesicle-media/toxin/supernatant combination was measured in three identical wells, to compensate for the small volume of vesicles. Positive and negative controls of vesicles were measured in the presence of diluted Triton X-100 in water and HEPES buffer, respectively.

2.5. UV-visible spectroscopy: The absorption spectra of control liposomes with varying degree of polymerization were analyzed with a SPECTROstar Omega micro-plate reader

(BMG Labtech) using a 96 well plate. 50 µl of liposomes were deposited in each well, and the respective absorbance at 37 °C was measured over a 400 - 700 nm wavelength range.

2.6. Data presentation: Fluorescent data is presented in standard response (%). The average and standard deviation of fluorescence release was used to calculate the fluorescent response (%) using the equation below:

$$\% \text{ Fluorescent response} = \frac{F_{final} - F_{initial}}{F_{Triton} - F_{initial}} \times 100\% \quad (2.1)$$

where F_{final} is the average fluorescence in bacteria/HEPES solution after 24 hours, $F_{initial}$ is the average fluorescence in bacteria/HEPES solution at the onset of the experiment and F_{Triton} is the average fluorescence following disruption by the detergent Triton X-100 solution after 24 hours and F_t is the specific fluorescence value at a specific time point.

UV-Vis spectroscopic data of control liposomes, before and after incubation at 37 °C for 16 hours, is presented either in the form of overall absorption spectra or specific absorbance peaks at wavelengths of 650 nm (blue) and 550 nm (red).

2.7. Pathogenic bacteria and their culture supernatant: Two strains of pathogenic bacteria, *P. aeruginosa* PAO1 and *S. aureus* LAC, were cultured in LB and TSB media, respectively. Both bacteria were grown from a single colony inside a shaker incubator at 37 °C for 18 hours. The overnight culture was centrifuged for 15 minutes at 5000 rpm, the supernatants were filter-sterilized through 0.22 µm porous filters and stored -20 °C until further use.

3. RESULTS AND DISCUSSION

3.1. Optimization of cholesterol and TCDA in liposomes: Cholesterol is an essential lipid component of the eukaryotic cell membrane, playing an important role in modulating biophysical properties of the lipid bilayer, such as fluidity and orientation of lipids[33] and

permeability of the plasma membrane.[23,31] *In vitro* studies of liposomes containing cholesterol provide the present knowledge of cholesterol-lipid interactions, allowing the temporal control of cholesterol over stability of liposomes.[32] The effect of cholesterol in lipid-PDA liposomes was studied by formulating DPPC and DSPC liposomes containing 10, 20 and 30 % cholesterol with 25 % TCDA (Figure 1).

The optimum percentage of TCDA in phospholipid liposomes was previously investigated, with liposomes containing 25 % TCDA chosen as an optimum composition, for their stability and sensitivity to bacterial toxins and supernatants.[20] The results indicated that the passive leakage was notably reduced in liposomes containing TCDA, with the exception of the DPPC liposomes containing 10 % cholesterol (figure 1(i)). Polymerized TCDA restricted the translational mobility of lipids, thereby decreasing the permeability of lipid bilayer compared to liposomes without the TCDA (figure 1(ii)). However liposomes (especially DSPC) containing TCDA showed lower sensitivity to bacterial supernatant, indicating a possible counterproductive effect of TCDA liposome toxin-lipid interaction (figure 1(iii)). The sensitivity of liposomes without TCDA was not much affected, as the trend of cholesterol-dependent toxin sensitivity was generally maintained (figure 1(iv)). Thus liposomes with a 20 % cholesterol and 25 % TCDA composition were chosen for their optimum stability and sensitivity, and subsequently used in all liposomes studied in the present work.

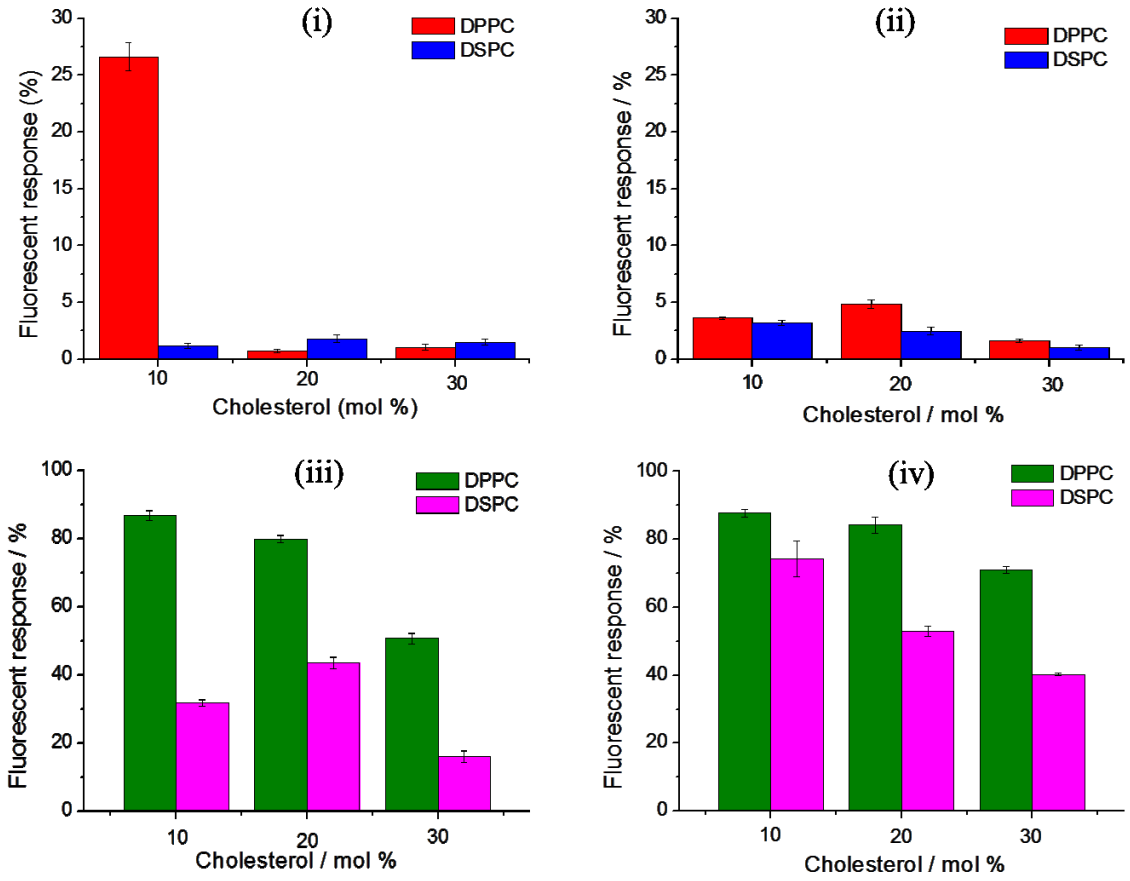

Figure 1 Cholesterol dependent stability of DPPC and DSPC liposomes containing 25 % TCDA in HEPES buffer, along with sensitivity to bacterial supernatant (*S. aureus* strain MSSA 476); stability of liposomes (i) with, (ii) without TCDA; and sensitivity of liposomes (iii) with, (iv) without TCDA

3.2. UV polymerization of control lipid-TCDA liposomes: The polymerisation of control liposomes, which contained only HEPES buffer, were studied with respect to varying low temperature annealing times, examined by measuring the absorbance spectrum over a wavelength range of 400 - 700 nm. The absorbance peak associated with 650 nm (blue) was used to correlate the extent of TCDA polymerization within the liposome bilayer. No polymerization occurred when liposomes were exposed to UV light at room temperature (figure 2(b)), possibly due to the lateral disorder of TCDA molecules at room temperature.[11]

Regardless of the type and chain length of surrounding lipids, the liquid-disorder state of the bilayer may disrupt the lateral packing of TCDA monomers, which could subsequently limit the polymerization upon UV activation. The characteristic pale blue colour, accompanied by a peak absorbance at around 0.25 was, however, clearly observed in DSPC liposomes which were polymerized after having been stored at 4 °C for 24 hours (figure 2(c)).

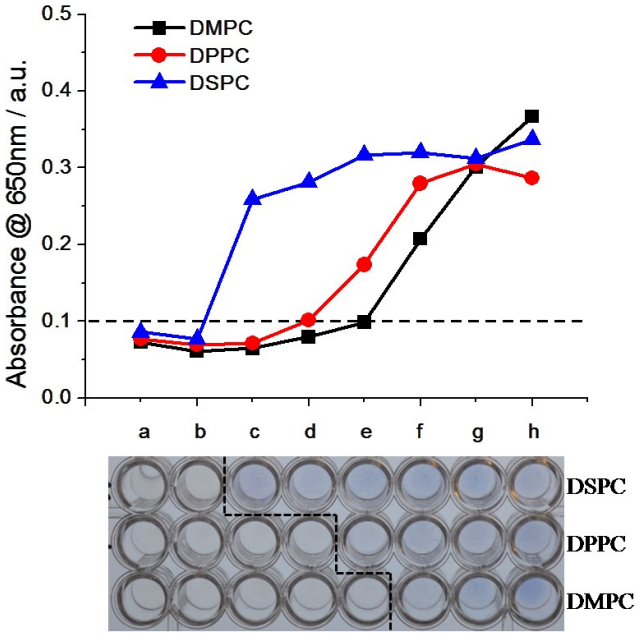

Figure 2 Degree of UV polymerization of "empty" liposomes (dashed line partitioned below and above absorbance of 0.1) and the respective absorbance peaks at 650 nm with liposomes at the following stages: (a) no polymerization, (b) polymerization at 25 °C directly after production of liposomes, and polymerization after (c) 1, (d) 2, (e) 3, (f) 4, (g) 5 and (h) 6 days of storage at 4 °C.

For effective UV-polymerization to occur, PDA domains in liposomes must meet two critical conditions. Firstly, PDA monomers must align laterally in highly ordered lattice domains.[12] Such domains may only exist in the gel phase below the main transition temperature, either in lipid-PDA bilayer with a major PDA content,[19] or similar chain length of PDA with

surrounding lipids in the bilayer membrane (figure 3(i)). Secondly, PDA monomers must be tilted within the bilayer, which brings the two upper and lower triple bonds of adjacent PDA molecules into close proximity, forming 1,4-polymerization of diacetylenes (figure 3(ii)).[11] Without meeting these requirements, polymerization may not occur.

Low temperature annealing of the liposomes brings the liquid-disorder state of bilayer membrane to the lipid gel phase, in which lipids and TCDA molecules form the closely packed domains with their chains in fully extended form. Such highly ordered domains are important in a lattice geometry, bringing the TCDA monomers in lateral close proximity, creating conditions for successful photo-polymerization[11,12] (figure 3(iii)). A TCDA monomer has 22 carbon atoms in its hydrocarbon tail with 2 triple bonds at C10 and C12 with respect to the OH head group (figure 3(ii)). The intermolecular section of the extended triple bonds is tilted at, 30° from the molecular axis, resulting in an apparently shorter TCDA molecular length when viewed perpendicular to the bilayer. After polymerization the half-bilayer thickness of TCDA domains is further decreased, as 1,4-photopolymerization utilizes two carbons per molecule between two adjacent monomers to form a polymerized bridge. The overall result is a TCDA molecule which is similar in chain length to that of the DSPC hydrophobic tail (figure 3(i)), emphasizing the important role played in effective polymerization of lipid-PDA liposomes by neighbouring lipids which have similar chain lengths to TCDA after polymerization. Similar chain length and low temperature annealing possibly provides effective UV polymerization of DSPC liposomes, as evidenced by the appearance of the characteristic blue colour over their shortest annealing time, in contrast to its absence in the DMPC and DPPC liposomes (figure 2(c)).

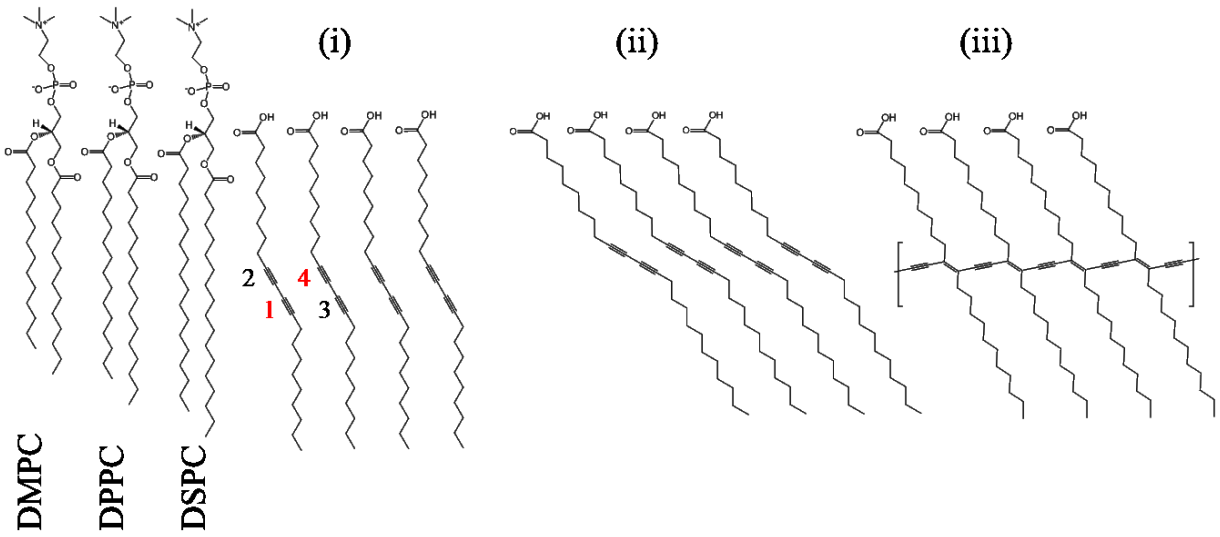

Figure 3 Representations of differing chain lengths of (i) TCDA with DMPC (C14:0), DPPC (C16:0) and DSPC (C18:0) in all-*trans* configuration, (ii) TCDA lattice domain at a 30° tilt to the bilayer, and (iii) after 1,4-photopolymerization at a 30° tilt orientation.

Interestingly, DMPC and DPPC liposomes exposed to the same low temperature annealing, up to 2 and 3 days, respectively, showed no sign of polymerization (figure 2(d and e)). In general, it was observed that the shorter the lipid chain length, the longer the time period required to effect TCDA polymerization. There is no credible rationale given in the literature to explain this observation. It could, however, be due to the hydrophobic mismatch and the smaller chain-tilt-angle of shorter lipids. The phospholipid chain tilt within bilayers is a well-known natural solution to resolve a simple packing problem, due to the space required by a bulky polar head group (S) being larger than the area occupied by the hydrophobic tails (2Σ) of each lipid molecule (i.e. $S>2\Sigma$).[22] In phosphatidylcholine the bulky head group takes up approximately 50 $Å^2$ which is not compensated for by the space of its two acyl chains, i.e. 38 $Å^2$.[23] In a liquid-disorder phase, the packing problem is naturally resolved by *gauche* configurations (chains disorders) of lipid acyl tails.[22] There is, however, an issue in the gel phase where lipids are in all-*trans* configuration, and closely packed with relatively strong hydrophobic interactions amongst the acyl tails. In this case the packing difficulties are only

resolved by the tilting of acyl chains, and the cross-sectional area of lipid tails in bilayer normal is increased in order to be compatible with that of the head group.[22,23]

The tilt angle of a phospholipid is chain length-dependent. In DMPC acyl chains are tilted by approximately 12°, while in DPPC they are tilted by as much as 30° away from the bilayer.[22, 23] The chain tilt angle of DSPC in a lipid bilayer is not reported but could be hypothetically estimated to be larger than 30°. The chromatic and absorbance results depicted in figure 2 suggest that DSPC, possessing a longer chain length and a possibly larger tilt angle, provide the TCDA monomers with the closest proximity orientation to effect 1,4-photopolymerization. In contrast, DMPC and DPPC liposomes did not achieve the optimum orientation for TCDA polymerisation within 24 hours of low temperature annealing (figure 2(c)). Nevertheless, a degree of TCDA polymerization was observed in these liposomes after continuous low temperature annealing over a period of days - however the maximum degree of polymerization was not achieved within 5 days (figure 2).

3.3. UV polymerization of fluorescence-containing lipid-TCDA liposomes: UV polymerization was similarly carried out on the control liposomes, however absorbance measurements via UV-Vis spectroscopy were not carried out due to fluorescent interference from carboxyfluorescein. DPPC liposomes exposed to varying UV doses, from 30 to 90 mW cm^{-2}, demonstrated a dose-response colour transition: from yellow to green (figure 4 (i) to (iv)). The optimal dose was shown to be 90 mW cm^{-2} as the chromatic response of liposomes displayed no further change when exposed to doses of up to 150 mW cm^{-2} (figure 4(iv)). No chromatic transition took place before and after UV polymerization in freshly prepared liposomes without low temperature annealing, (figure 4a and 4b respectively). The chromatic colour transition of liposomes after UV polymerization (figure 4a to 4h) strongly agreed with the absorbance results of control liposomes, shown in figure 2. Polymerized DSPC vesicles

converted to green after 24 hours of low temperature storage, however DPPC and DMPC vesicles required 3 to 5 days, respectively, to achieve a similar degree of polymerization.

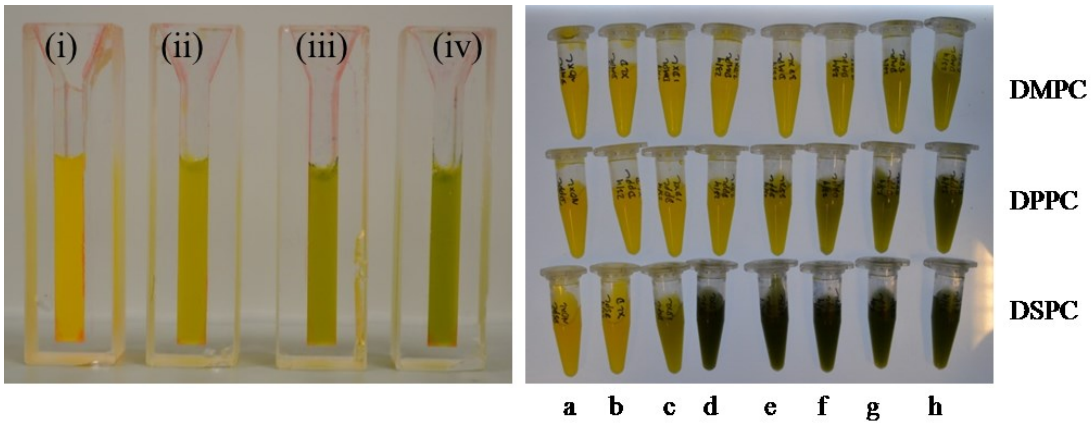

Figure 4 Degree of polymerization of DPPC liposomes (after 5 days of storage at 4 °C) with UV doses of (i) 0, (ii) 30, (iii) 60 and (iv) 90 mW cm^{-2} (left), and colourimetric expression of UV polymerized liposomes (right) with (a) no polymerization, (b) polymerized at 25 °C directly after production of liposomes, and polymerization after (c) 1, (d) 2, (e) 3, (f) 4, (g) 5 and (h) 6 days of storage at 4 °C.

High UV doses (up to 8.58 W cm^{-2}) were utilized to examine the effect of excessive polymerization on the stability of the liposomes. DMPC liposomes, polymerized above 480 mW cm^{-2}, exhibited notable leakage of carboxyfluorescein (figure 5). Intriguingly, DPPC and DSPC liposomes were stable, exhibiting minimal passive leakage regardless of exposure to excessive UV doses. When exposed to increasing UV doses at 37 °C, the liposomes changed colour from green through pink to red, with no significant leakage observed over a two hour period, particularly in the case of DSPC liposomes. When the control liposomes were subjected to the same excessive UV exposure, the absorbance peak at 650 nm (blue) first appeared at 90 mW cm^{-2}, followed by the progressive appearance of peaks at 560 nm (pink/red) with higher UV doses (figure S1 and S2 in the supplementary information). Size analysis of these liposomes, by means of Dynamic Light Scattering (DLS), showed no

significant change, affirming there was neither formation of aggregations nor reduction to liposomal micelles due to excessive UV exposure (data not shown). Therefore, rapid leakage induced by excessive polymerization of DMPC liposomes may be due to hydrophobic mismatch boundaries between lipid and irreversibly stress-induced TCDA domains, which in contrast show no effect on passive leakage of DPPC and DSPC liposomes (figure 5).

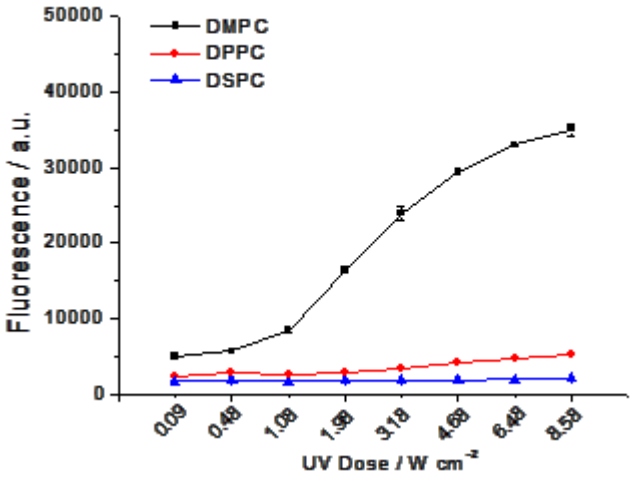

Figure 5 The effect of extended liposome UV polymerization on passive leakage of carboxyfluorescein in HEPES at 37 °C.

3.4. Effect of UV polymerization on stability of liposomes: The formation of the TCDA polymer backbone improves the rigidity of the polymer domains within the lipid bilayer. This stable polymer lipid bilayer is then surrounded by lipid domains which act as windows for biologically active interfaces.[18] The effect of polymerization on stability was examined by monitoring the passive leakage of carboxyfluorescein from liposomes with different degrees of UV-crosslinking (figure 4(right)). These studies were carried out in HEPES buffer incubated at 37 °C for both short and long time periods, of a day and a week, respectively. The short-term stability of liposomes is depicted in Fig 6(i) with net fluorescent release plotted over a 24 hour period. DMPC liposomes exhibited considerable leakage despite

having achieved the highest degree of polymerization after 6 days (figure 2(h)). Minimal passive leakage was observed in DPPC and, most especially, in DSPC liposomes. Relatively higher leakage was observed in liposomes with intermediate polymerizations. This could be due to the poor efficiency of polymerization when TCDA monomers were not yet properly aligned. Random orientation of the TCDA monomers may undermine effective polymerization during UV exposure, giving rise to higher fluorescence leakage. This suggests that a maximum TCDA polymerization, exhibited by a vivid colour transition, may not necessarily provide a stable state, unless acyl chain compatibility is achieved between the lipids and the TCDA domains. Interestingly the liposomes exhibited "a general trend" of decreasing leakage with respect to an increasing degree of polymerization (see figure 6). This is further illustrated by a distinct chromatic transition, seen in figure 4. This supports our hypothesis that the degree of TCDA polymerization improves the liposome stability, in terms of minimizing the passive leakage under the experimental conditions.

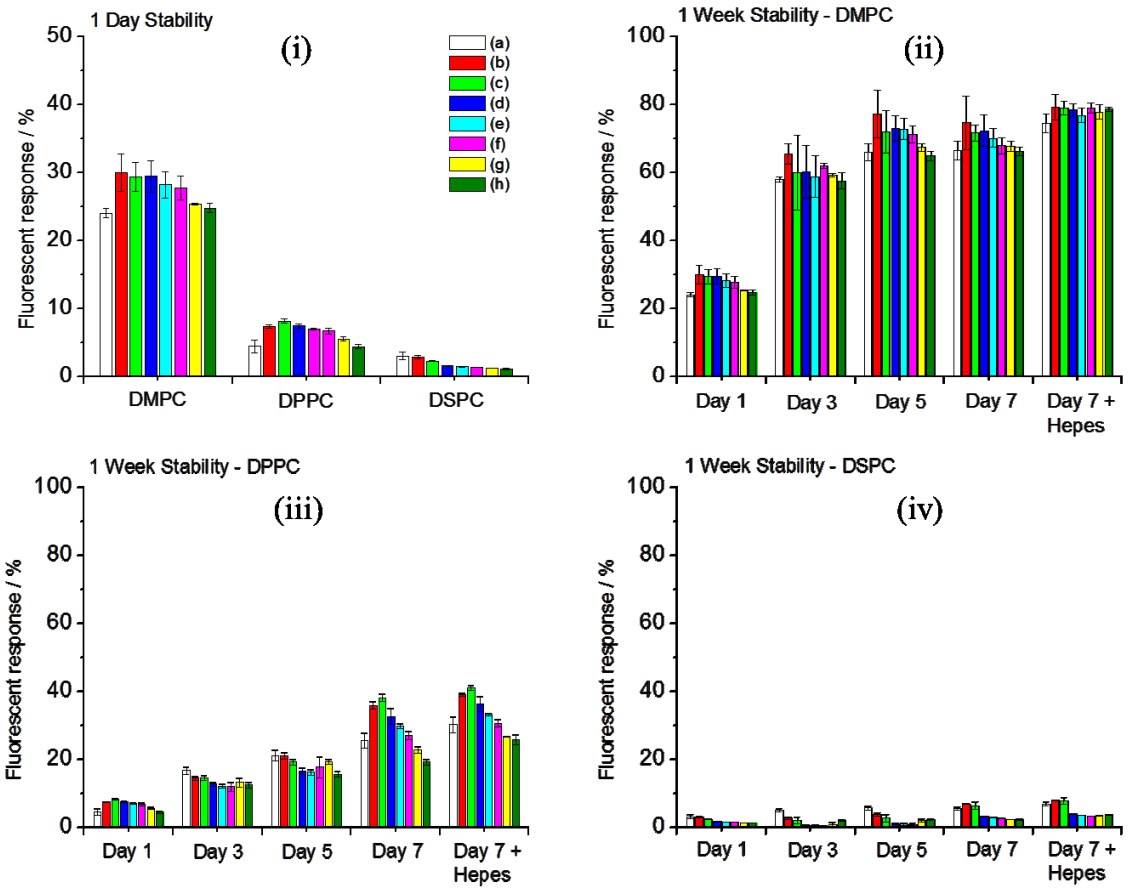

Figure 6 Short-term (24 hours) and long-term (7 days) liposome stability (in HEPES buffer at 37°C) under the conditions of: (a) no polymerization, (b) polymerized at 25 °C directly after production of liposomes, and polymerization after (c) 1, (d) 2, (e) 3, (f) 4, (g) 5 and (h) 6 days of storage at 4 °C.

A similar stability trend was observed in the long-term studies (figure 6(ii to iv)). All liposomes subjected to 37 °C incubation persistently displayed a gradual release of fluorescence. Significantly, these occurred at different rates. Within 3 days, DMPC liposomes leaked up to 60% of their fluorescent pay-load, while less than 20% leakage was observed in the DPPC liposomes. In contrast, DSPC liposomes showed negligible leakage within the same time period. The effect of the higher degree of polymerization on passive leakage was clearly observed in DPPC liposomes on day 7, with liposomes polymerized after 6 days

releasing 50% of the fluorescence payload w.r.t. those polymerized after 24 hours (figure 6(iii)). The stability of liposomes after 7 days was tested by addition of HEPES buffer, followed by Triton X-100 as a positive control. DMPC liposomes exhibited up to 80% leakage after day 7, while DPPC and DSPC liposomes only leaked up to 40% and less than 10%, respectively. The DSPC liposomes polymerized after more than 24 hours of storage provided the best 7-day stability (figure 6(iv)).

3.5. Dose-responses of liposomes with bacterial toxins/supernatants: The detection of clinically significant microorganisms, namely *S. aureus* and *P. aeruginosa***,** by lysis and fluorescent response of these PDA-liposomes is of major interest.[24] Based on the vast array of virulence factors produced by these two organisms, we chose two exoproteins for further study; the small amphipathic alpha helical delta toxin with its lytic mode of action against lipids contained within the target cell membranes and the glycolipid rhamnolipids.[25] The δ-toxin, secreted by *S. aureus* strains interacts with the cell membrane, with effects dependent on the peptide concentration, ranging from lipid domain perturbation at low concentration to detergent-like solubilisation at above threshold concentration, leading to the *in-vivo* lysis of eukaryotic cells.[26] Rhamnolipid, considered a bacterial bio-surfactant, is a key virulence determinant of *P. aeruginosa* and is responsible for the inhibition and lysis of epithelial cells *via* a detergent mode of action.[27] To investigate the lypolytic response and the pathogenicity of various virulence factors in supernatant, liposomes were tested with cultured supernatants of *P. aeruginosa* PAO1 and *S. aureus* LAC strains. Cultured supernatants were tested with liposomes, in order to investigate the pathogenicity of various virulence factors involved in supernatant to lipolytic response. The limit of detection (LOD) was defined as the supernatant/toxin concentration providing the normalized response of 20 to 40 % approximately (figure 7).

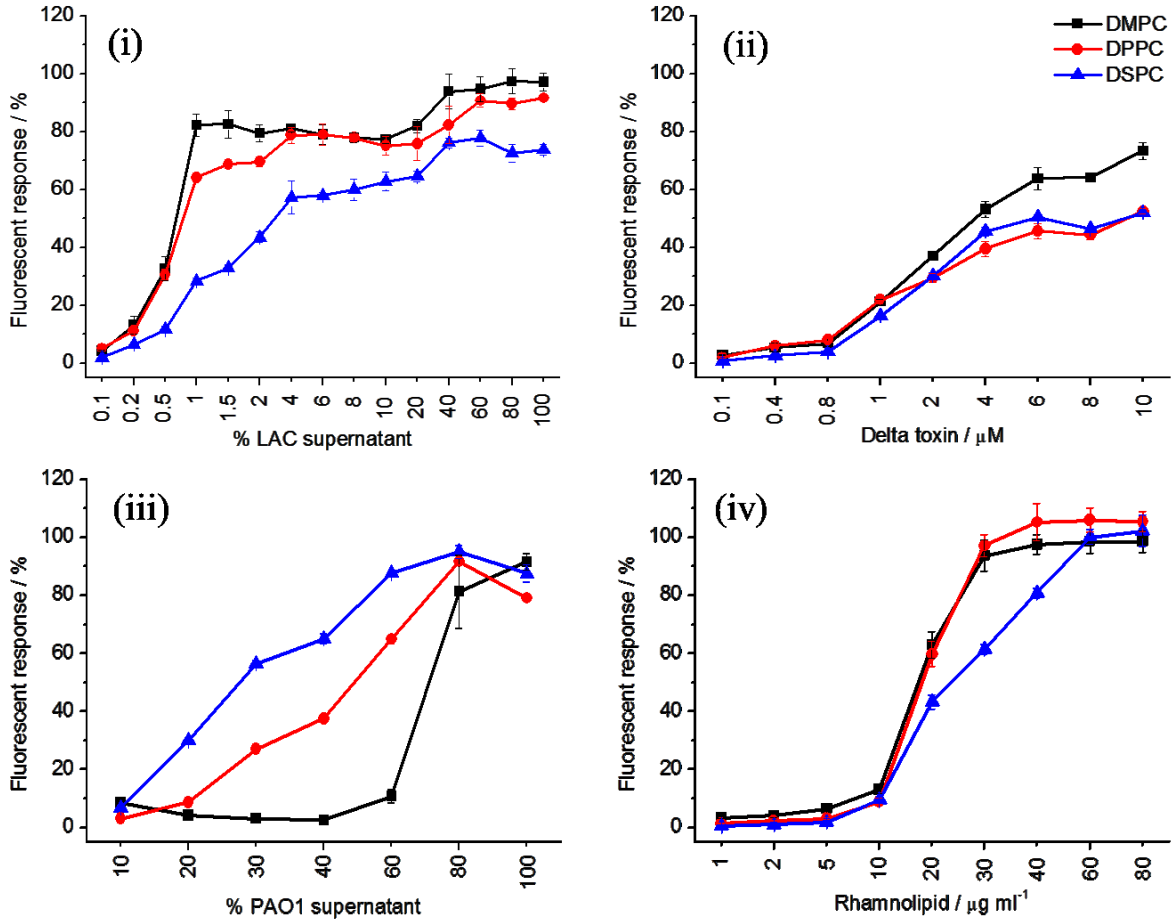

Figure 7 The fluorescent responses of liposomes, UV polymerized after 24 hours of low temperature annealing, to bacterial supernatants and purified toxins; (i) *S. aureus* LAC supernatant, (ii) δ-toxin, (iii) *P. aeruginosa* PAO1 supernatant, and (iv) rhamnolipid.

Figure 7 presents the experimental dose-response curves of liposomes with respect to the sequential dilutions of supernatants and purified toxins in a descending order. The fluorescent responses to higher concentrations of PAO1 supernatant and rhamnolipid were slightly more than 100 %. This indicates that some bacterial lysins presumably have a stronger lypolytic effect than liposomes solubilized by Triton X-100.[28,38] The response of DMPC and DPPC liposomes to LAC supernatant were as low as 0.5 %, while 1 % of the same supernatant was required to yield the same fluorescent response in DSPC liposomes. The secretion of virulence factors in bacteria is known to be density-dependent, and that in *S. aureus* it is

controlled and regulated by the accessory gene regulator (*agr*), utilizing the quorum sensing of cell-cell communication.[34] The Agr system in *S. aureus* is solely responsible for encoding the genes that control the production of virulence factors, including δ-toxin and phenol-soluble modulins (PSMs), alongside some non-specific toxins such as protein A.[35,36] Binding and activation of δ-toxin onto lipid bilayer is the function of toxin concentration, and is dependent on the membrane fluidity.[26] We observed that DSPC liposomes, with a relatively higher degree of polymerization (figure 4(c)) in contrast to DMPC and DPPC liposomes, exhibited a slightly lower sensitivity to LAC supernatant (figure 7(i)).

Interestingly, the sensitivity of liposomes to PAO1 supernatant was in reversed order, compared to that of the LAC supernatant; with DSPC and DMPC liposomes displaying the highest and the lowest responses, respectively, to the PAO1 supernatant at around 60 % (figure 7(iii)). This observation led us to postulate that the virulence factors belonging to the PAO1 supernatant may independently act on the lipid bilayer membrane, regardless of their polymerized conditions. All liposomes, however, displayed the response to rhamnolipid as low as 20 μg ml^{-1} (figure 7(iv)). This falls within the range of the cytolytic dose of rhamnolipid on erythrocytes i.e., 12 ~ 26 μg ml^{-1}, as detailed in a previous report.[29] The notable response of liposomes to supernatants and toxins, as judged from the experimental dose-response curves in figure 7, were used for further examination of liposomes produced with varying degree of polymerization.

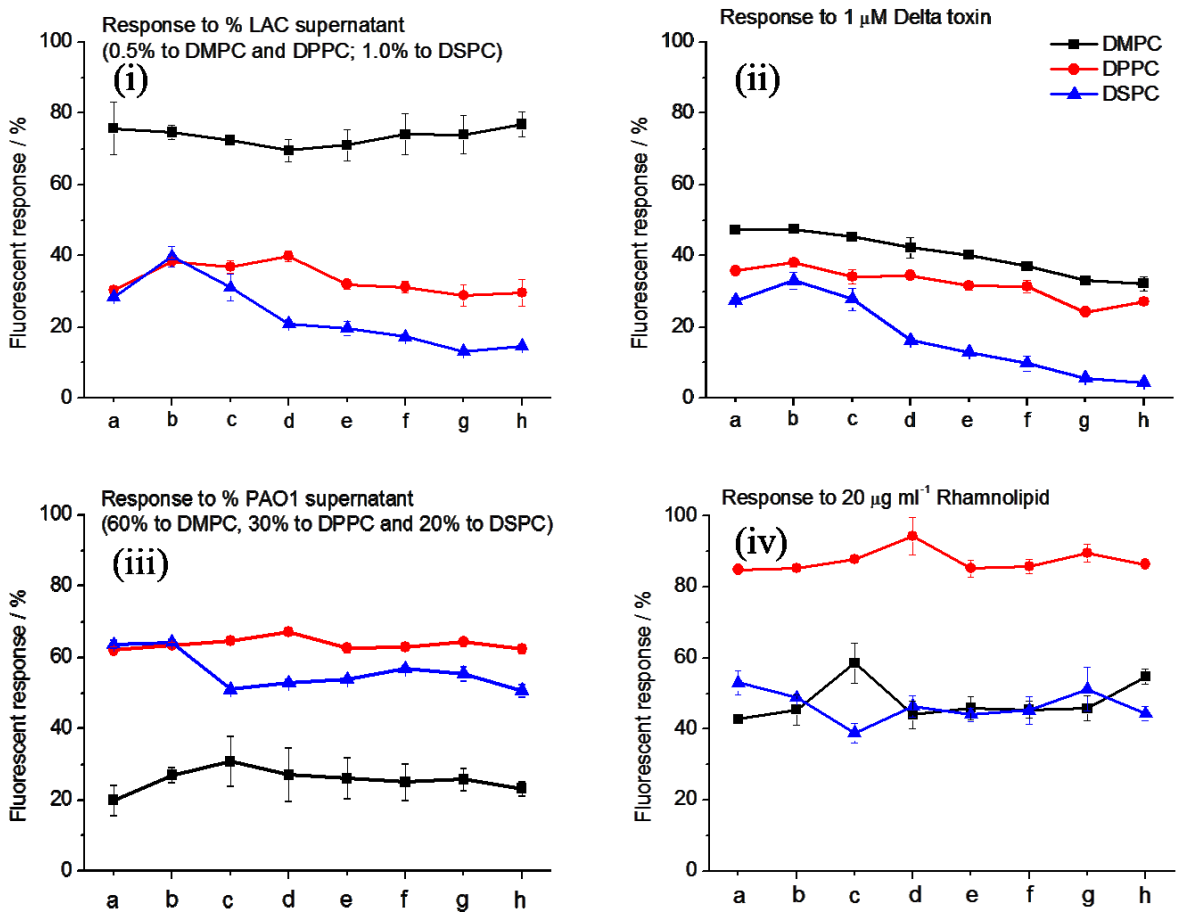

Figure 8 The fluorescent response of liposomes with (i) 0.5 to 1.0 % LAC supernatant, (ii) 1 μM δ-toxin, (iii) 20 to 60 % PAO1 supernatant and (iv) 20 μg ml^{-1} rhamnolipid (liposomes under the conditions of: (a) no polymerization, (b) polymerized at 25 °C directly after production of liposomes, and polymerization after (c) 1, (d) 2, (e) 3, (f) 4, (g) 5 and (h) 6 days of storage at 4 °C).

3.6. Effect of liposome polymerisation on toxins/supernatants: Figure 8 displays the fluorescent responses of polymerized liposomes with respect to the lysins at specific concentrations, judged from the dose-response curves in figure 7. DMPC liposomes displayed a fairly consistent sensitivity to LAC supernatant without being affected by the polymerized conditions of TCDA (figure 8(i)). DSPC liposomes, however, showed the lowest response to LAC supernatant, displaying a trend of decreasing sensitivity with respect to

increasing TCDA polymerization (figure 8(i)). Similar responses were observed in tests on δ-toxin at a concentration of 1 μM. The order of sensitivity of liposomes to δ-toxin was maintained, as indicated in the LAC supernatant test, with a relatively higher sensitivity being displayed by DMPC, followed by DPPC and DSPC liposomes. Interestingly, all the liposomes exhibited a similar trend of decreasing response with respect to an increasing degree of TCDA polymerization (figure 8(ii)).

In contrast to LAC supernatant, 60 % of PAO1 supernatant was not sufficiently lipolytic to DMPC liposomes, whereas DPPC and DSPC liposomes were lysed using only 20-30 % of the same supernatant (figure 8(iii)). Remarkably, all liposomes showed consistent responses despite the varying degree of TCDA polymerization. A slightly lowered response in polymerized DSPC liposomes was observed, however the polymerisation conditions did not seem to notably affect the sensitivity to PAO1 supernatant. It has been reported that the rhamnolipid was among the primary virulence factors of *P. aeruginosa* strains that are responsible for chronic respiratory and pulmonary infections.[27,29] The tight junctions of epithelial membrane were disrupted by the purified rhamnolipid, thereby promoting the paracellular invasion of rhamnolipid-deficient *P. aeruginosa*.[27] The mono-rhamnolipids caused the extensive blebbing of the human macrophage plasma membrane[29] while the di-rhamnolipids, with larger rhamnose moieties, destabilized the lipid membrane.[38,39] We hypothesize that the rhamnolipid has to be the main lytic agent in POA1 supernatant and is responsible for the lipolysis against the target cell membrane by the mode of action of biosurfactant. This could be explained by the *in-vivo* rhamnolipid that acts to stimulate the biodegradation rate of acyl chains in the eukaryotic cell membrane for the uptake of hydrocarbons by *P. aeruginosa*.[30,40] Based on these evidences, rhamnolipid is believed to be a primary virulence factor, being able to degrade the polymerized hydrocarbon chains, and induce the passive leakage of liposomes, regardless of the degree of polymerisation. This

hypothesis is supported by the liposomes tested with 20 μg ml^{-1} rhamnolipid (figure 8(iv)). Similarly to the PAO1 supernatant response, all liposomes displayed the same order of sensitivity to rhamnolipid, and did not appear to be affected by their polymerized conditions.

In summary, the sensitivity of liposomes to *S. aureus* LAC supernatant and δ-toxin was affected by the degree of TCDA polymerization, with the general trend being a lower response w.r.t. a higher degree of polymerization.[37] The polymerization of TCDA may restrict the overall fluidity of liposomes, thus improving the overall stability and minimizing the passive leakage of liposomes. This may, however, limit the non-specific binding and activation of toxins such as δ-toxin of *S. aureus*, onto lipid domains of the liposomes. In contrast, the sensitivity to rhamnolipid and *P. aeruginosa* PAO1 supernatant were not adversely affected.[37]

4. CONCLUSION

The effect of polymerization of lipid-PDA liposomes on stability and sensitivity to bacterial toxins was studied. The sensitivity to two primary cytolytic toxins and cultured supernatants of *S. aureus* LAC and *P. aeruginosa* PAO1 were examined. UV absorbance data indicated that the degree of polymerization was strongly influenced by the lipid chain tilt and hydrophobic mismatch of the surrounding lipids with TCDA domains in gel phase, and the duration of low thermal annealing at below the lipid phase transition temperatures. The lipids with shorter chains, such as DMPC, required lengthier annealing than the relatively longer chain lipids, such as DSPC, to yield the optimum polymerization. In general, minimum leakage with an acceptable long-term stability was achieved by increasing degree of TCDA polymerization. The stability of liposomes, with lipids longer than 14 carbons, was not significantly affected by the excessive polymerization in extreme UV doses of up to 8.58 W cm^{-2}.

The liposomes, however, displayed different responses to sequentially diluted cultured supernatants of LAC and PAO1 strains, possibly due to several virulence factors involved in bacterial cultured media. Tests on liposomes at their respective LOD to toxins and supernatants indicated that the higher degree of polymerization only slightly decreased the relative sensitivity to LAC supernatant and δ-toxin, while rhamnolipid and PAO1 supernatants were not notably affected by the degree of TCDA polymerization. The results discussed in this work highlighted the important role of lipids in PDA liposomes in the development of pathogen sensors - relying not only on the general chromatic colour indication, but also the integration of lipids to render the PDA liposomes more robust, yet maintaining a reasonable membrane fluidity to facilitate their interaction with target microbial agents.

ACKNOWLEDGEMENT

This work was financially supported by the UK EPSRC Healthcare partnership programme and the European Commission's 7th Frame-work programme via the EC-FP7 consortium project no. 245500 *Bacteriosafe*.

SUPPORTING INFORMATION

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