**Polyethylene glycol lipid-shelled microbubbles:**

**abundance, stability and mechanical properties**

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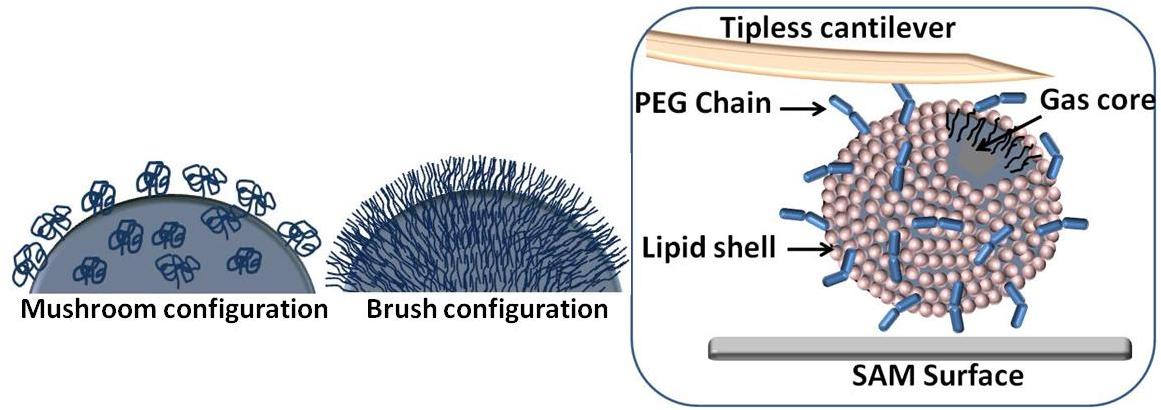
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**Running Title:** Effect of PEG on lipid microbubble formation and properties

**Abstract**

Poly-ethylene glycol (PEG) is widely used on the outside of biomedical delivery vehicles to impart stealth properties. Encapsulated gas microbubbles (MBs) are being increasingly considered as effective carriers for therapeutic intervention to deliver drug payloads or genetic vectors. MBs have the advantage that they can be imaged and manipulated by ultrasound fields with great potential for targeted therapy and diagnostic purposes. Lipid-shelled MBs are biocompatible and can be functionalised on the outer surface for tissue targeting and new therapeutic methods. As MBs (MBs) become a key route for drug delivery, exploring the effect of PEG-ylation on the MB properties is important. Here we systematically investigate the effect of PEG-lipid solution concentration ranging between 0-35 mol% on the formation of MBs in a microfluidic flow-focussing device. The abundance of the MBs is correlated with the MB lifetime and the whole MB mechanical response, as measured by AFM compression using a tip-less cantilever. The maximal MB concentration and stability (lifetime) occurs at a low concentration of PEG-lipid (~5 mol%). For higher PEG-lipid concentrations, the lifetime and MB concentration decrease, and are accompanied with a correlation between the predicted surface PEG configuration and the whole MB stiffness, as measured at higher compression loads. These results inform the rationale design and fabrication of lipid-based MBs for therapeutic applications and suggest that only relatively small amounts of PEG incorporation are required for optimising MB abundance and stability while retaining similar mechanical response at low loads.

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**1 Introduction**

The application of microbubbles (MBs) as drug delivery vehicles requires them to have long *in vivo* lifetimes. This is typically achieved via the use of steric stabilizers similar to those used in liposomal delivery, i.e. by incorporation of poly-ethylene glycol (PEG) chains on the outer surface. Typical MBs are gas filled spheres, 0.5-8 µm in diameter, with a phospholipid shell at the gas-liquid interface [[1-3](#_ENREF_1)]. PEG chains are widely used in the pharmaceutical industry, due to their ability to form a steric barrier and prevent coalescence [[4](#_ENREF_4), [5](#_ENREF_5)]. They can also be used as a conjugating agent by adding targeting ligands to the free end of the chain [[6](#_ENREF_6)]. PEG chains covalently bonded to phospholipids are integrated into the MBs phospholipid shell to reduce the immunogenic response by creating a barrier that prevents the adhesion to opsonins in the blood, such that the MBs remain invisible to the phagocytic cells, giving stealth properties [[6](#_ENREF_6), [7](#_ENREF_7)]. Adding a functional group to the end of the PEG chain allows binding of other molecules or assemblies, such as antibodies or liposomes to the MB surface for targeting and drug delivery purposes [[6-9](#_ENREF_6)].

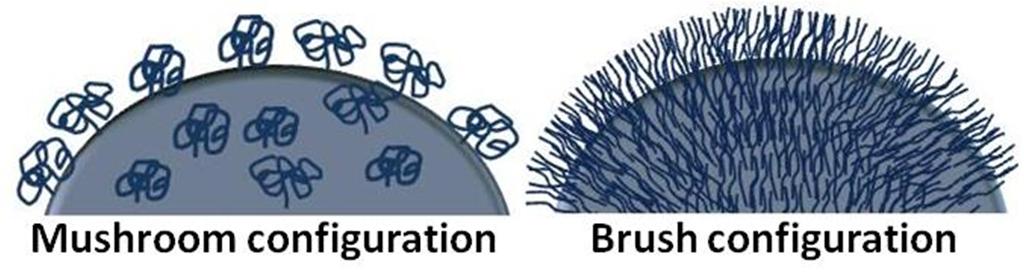
PEG chains are being increasingly used for stealth biomedical applications and different drug delivery systems due to their physical and chemical properties to prevent recognition and surface adhesion. This field involves studies for either enhancing the circulation lifetime of the system or for increasing the functionality by attaching molecules to it [[4](#_ENREF_4), [5](#_ENREF_5)]. The effect of PEG chain molecular weight [[10](#_ENREF_10), [11](#_ENREF_11)] and concentration [[12](#_ENREF_12)] on liposomal permeability, compressibility and packing of the lipid bilayer [[13](#_ENREF_13), [14](#_ENREF_14)] has been investigated.

Figure 1: Schematic diagram representing the PEG configurations at low and high surface coverage densities

The PEGylation density is an important factor for defining the PEG conformation achieved and depends upon parameters such as the Flory radius of the grafted PEG (RF) (eq1), the distance between PEG chains (D) (eq2) and the length/thickness of the grafted PEG (L) (eq3) [[15](#_ENREF_15), [16](#_ENREF_16)].

(1)

(2)

(3)

Where, a, is the size of the monomer (=0.35nm), N, is the number of monomers per chain (=45), Alipid, is the area occupied per lipid which varies between 0.48 and 0.67 nm2 depending on whether the lipids are in gel or liquid phase[[17](#_ENREF_17), [18](#_ENREF_18)], and m, is the mole fraction of PEG incorporated in the shell [[16](#_ENREF_16), [19](#_ENREF_19), [20](#_ENREF_20)].

In 1987 De Gennes [[21](#_ENREF_21)] referred to two main configurations (Figure 1), mushrooms at low PEG coverage when D>RF, and by increasing the density of PEG coverage the configuration gradually changes to form brush layer with the chains extended when D<RF. In the brush regime Damodaran et al [[22](#_ENREF_22)] also distinguished a dense brush configuration when L>2RF.

Since MBs are being increasingly studied for localised drug delivery, understanding the effect of PEGylation on their physical and biological properties is important. The effect of the brush layer architecture on ligand conjugation was investigated [[6](#_ENREF_6), [23](#_ENREF_23)] giving evidence for the protective role of PEG brush, inhibiting large molecules from reacting with surface functional groups. The effect of shell properties, specifically PEG concentration and molecular weight, on the cavitation threshold has been investigated by Wrenn et al, [[24](#_ENREF_24)] using PEG2000 and PEG6000. These data show that increasing concentration and molecular weight of PEG improves the bubble resistance to destruction, with the PEG2000 not showing significant difference in the range of 0.5-3 mol%. On the other hand, studies of the MB lifetime for equivalent molecular weight of PEG2000 and PEG40 sterate have shown that the resistance of the shell to gas permeation increases 10-fold for PEG2000 as a result of absence of the acyl chain from PEG40[[7](#_ENREF_7), [8](#_ENREF_8)].

Recent studies on a variety of MB shell architectures demonstrate that the shell composition and structure influences the mechanical properties of the MBs as measured by AFM [[25-27](#_ENREF_25)]. The response of the MBs to an ultrasound (US) field is related to the mechanical properties of bubbles and the shell stiffness, so understanding and predicting MB and shell mechanical response is important for the rational design of MBs for therapeutic delivery. This is particularly relevant when US is used as both an imaging diagnostic tool and to control localised drug release from the MB vehicle.

MBs concentration, size distribution, lifetime and shell properties are all important aspects for their efficient use in therapeutic delivery, to enhance the ultrasound imaging contrast and increase the concentration of locally delivered drug without blocking blood vessels. This study investigated the effect of changing the PEG-lipid concentration 0-35 mol% in the shell of lipid encapsulated MBs. The MBs were produced on a microfluidic flow focussing device in a microspray regime [[28](#_ENREF_28)]. The MB concentration, size distribution and lifetime *in vitro* were all investigated along with the characterisation of their mechanical response usingatomic force microscopy (AFM). The main aim was to explore the effect of different PEG concentrations on the shell mechanical response under low and high deformation conditions. This will guide and inform the process of lipid-shelled MB production through selection of the proper constituents to yield selective properties for combined delivery and US imaging.

**2 Materials and Methods**

**2.1 Materials:**

The lipids in use throughout this study are 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl (polyethylene-glycol) -2000] (DSPE-B-PEG2000), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) (DOPE-B) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All lipids were dissolved in 50/50 chloroform/methanol. Neutravidin from (Invitrogen, Life Technologies, UK). Cell medium used is RPMI from Invitrogen, Life Technologies, UK with 10% (v/v) foetal calf serum (Sigma Aldrich, UK).

**2.2 Microbubble preparation and characterisation:**

A mixture of DPPC and DOPE (in the case of 0% PEG) or DSPE-B-PEG2000 with molar concentration 2, 4, 5, 10, 15, 25, and 35% was prepared as described previously [[28](#_ENREF_28)]. Briefly, the lipid mixture was dried under a steady stream of nitrogen gas on the vial walls. This dried film was then suspended in a solution containing 4 mg/ml NaCl and 1% glycerine (Sigma-Aldrich, St. Louis, MO, USA) to a final lipid concentration 1mg/ml. This solution was vortexed for 1 minute before being placed in ultrasonic bath for 1 hour. The lipid solutions were allowed to cool in the fridge for 10 minutes prior to use in the microfluidic MB maker.

The lipid suspension was introduced into a polycarbonate microfluidics chip Epigem (Redcar, UK). Perfluorocarbon gas (C3F8) gas pressure of 15 psi controlled by a Kukuke microprecision regulator (RS supplies, Leeds, UK) and liquid flow rate of 80 µl/min controlled by Aladdin AL 2000 syringe pump (World Precision Instruments, Stevenage, UK) were fixed for all bubble preparations.

For each MB population formed, a sample was diluted 10-fold to facilitate counting and sizing bubbles. 30 µl of MB solution was collected from the middle of the homogenous sample and introduced in a 50µm chamber on a glass slide. The MBs were allowed to rise for ~2 minutes before acquiring images. 20 images were collected for each sample, from which the concentration and size distribution were obtained.

**2.3 Lifetime testing:**

MBs lifetime was tested *in vitro* in conditions that resemble the physiological conditions. After MB production, 500µl MB solution was introduced in 500 µl cell medium and incubated in Accu block digital dry bath (Model D1100, labnet international, inc, USA) at 37 0C. The vial containing the sample was left opened exposed to air. 10 µl MB sample was collected every 10 mins for sizing and counting. A CCD camera (DS-Fi1 5Mega pixel, Nikon, Japan) and Nikon Elements Software-D (Version 3.2) were used for capturing all MB images. The images were analysed using Image J freeware (http://rsbweb.nih.gov/ij/) and statistically analysed using Origin Pro (Version 8.5).

**2.4 AFM mechanical testing:**

For AFM testing MBs were immobilised on a support surface, to allow the repeat measurements with the initial load at the pole of the MB, as described previously [[25](#_ENREF_25), [29](#_ENREF_29)]. Briefly, glass cover slips were coated with a 20 nm thick layer of gold with a 2.5 nm Cr adhesion layer by thermal evaporation (Edwards Auto 306). These surfaces were incubated overnight in a [90:10 mol.-%] 6-Mercaptohexanol (Sigma Aldrich,Poole, UK) : biotinylated-thiol (Asemblon, WA, USA)][[30](#_ENREF_30)] to form a biotinylated self-assembled monolayer (SAM). The biotinylated SAM surface was incubated with 0.1 µM Neutravidin for ~1 hr prior to adding the bubbles. The sample was then placed into contact with the bubble solution in an inverted configuration for 1hr to enable the buoyancy force to bring the MBs to bind onto the surface.

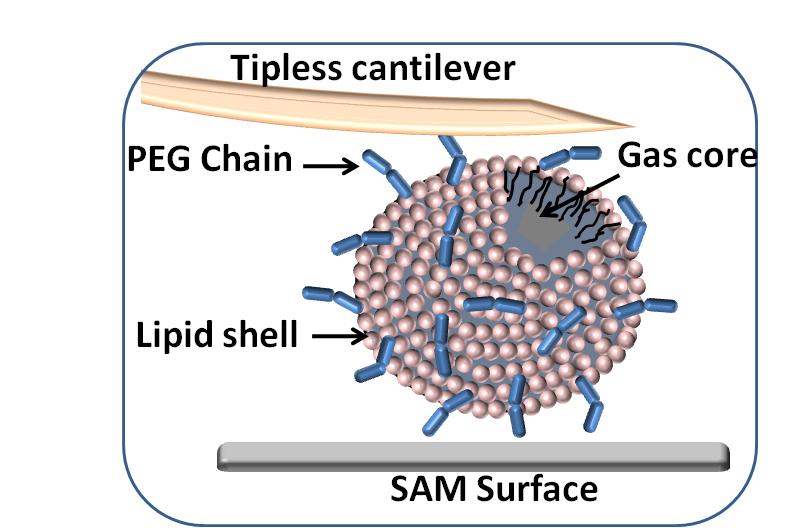
An Asylum Research MFP-3D AFM (Asylum Research, Santa Barbara, CA, USA), mounted with a tipless cantilever (Nanosensors, UK) was used for measuring MB stiffness. Spring constants of the cantilevers were calibrated using the thermal tuning method [[31](#_ENREF_31)] giving a range between 0.93-1 N/m. A single immobilised MB within a small size range (3-4µm) was selected for mechanical testing using the integrated optics of the AFM. The cantilever was then positioned above the pole of the bubble as illustrated in Figure 2, and force curves were acquired in continuous mode. For all bubble preparations, a range of maximum applied loads were used: 5, 10, 30 and 50 nN.

Figure 2: Schematic diagram to illustrate the configuration of the AFM experiment on a single bubble

The loading velocity was fixed to 3µms-1. For each bubble type the data presented is the average of 5 bubbles with more than 50 curves collected at each load to ensure reproducibility; Figure 3 presents a typical force-deformation curve on (a) plain SAM surface and on (b) a single MB to highlight the differences in mechanical response.

Compression values were derived from force-distance curves which were converted into Force-deformation curves by accounting for the movement of the cantilever base (Z-piezo motion) during the contact region, where the deformation or compression is the difference between Z-Piezo position and the deflection of the cantilever. This is achieved by determining the deflection sensitivity of the AFM detector for a given cantilever by taking a force-distance measurement on a non-deformable clean glass slide. MB stiffness was calculated from the gradient of the force-deformation data at given load for increasing applied force.

The gradient of the tangents at the different maximum applied loads was calculated to investigate the compression behaviour of bubble under increasing applied pressure. The final stiffness at a given load was determined from the tangent of the force-deformation curve over the last 50% of the force range for any given maximum load (dashed line in Figure 3b). This process was repeated for each PEG concentration, and for the control experiment using 10 and 35% DOPE-B (no PEG).

Figure 3: Typical force-deformation curves a) on the background SAM surface and b) on a MB. The SAM surface shows no deformation at all which is used as a control experiment to confirm that the deformation observed in (b) on bubble surface is related to the bubble only.

**3 Results:**

**3.1 Theoretical predictions and MBs characterisation:**

From equation (1) the area per head group for DPPC in the gel phase 0.48nm2 [[17](#_ENREF_17)] was rounded up to 0.5nm2, which gives a calculated Flory radius for PEG2000 to be 3.4 nm. Using equations (2), (3) we can calculate the expected chain spacing, D, and the PEG layer thickness, L (Table 1). From these values we see that we are in the mushroom configuration at 2% PEG, however at 4, 5% PEG we are very close to the boundary between the mushroom to brush transition. For the 10% and 15% PEG, the configuration is predicted to be in the brush regime, whereas for the 25% and 35% PEG it is in the dense brush regime.

Table 1: Calculated values for the distances between PEG chains (D) and the extended chain length (L) at each molar concentration used. Predictions for the PEG configuration use these parameters relative to the Flory radius (calculated assuming Alipid=0.5nm2).



A typical example of the MB population size distribution is shown Figure 4, giving an average diameter of 1.9±1.4 µm with a log normal fitting and no bubbles exceeding 7µm in diameter. The MB concentration and diameter at each PEG concentration is presented in the following section.

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Figure 4: Size distribution for one the bubble populations, with an inset showing an image of bubbles with bright light field.

**3.2 Effect of PEG on MBs Size and Concentration:**

The MB concentrations obtained under the same flow conditions were found to be dependent on the percentage of PEG2000. With no PEG attached to the lipid shell the average MB concentration was found to be 2.2x108 bubbles/ml (Figure 5). Gradual increase in PEG concentration from 2-5% resulted in an increase in the concentration of the MB population leading to a maximum of 10 fold increase at 5% PEG (Figure 5b). The addition of further PEG led to significant decrease in MB concentration until at 35% PEG, the MB concentration was ~ 4.4x108 MBs/ml.

Bubble size was found to vary around a mean diameter of ~ 1.9±1.4 µm. An increased amount of foaming was observed as the PEG concentration in the bubble mixture was increased (Figure 5c). The foaming did not affect the measured bubble size but perhaps helps to explain the gradual reduction in the abundance of MBs produced.



Figure 5: The effect of PEG density on MB concentration and size. (a) Numerical values for MB concentrations. (b) MB concentration as a function of %PEG. Error bars represent the variations between 4 preparation repeats. (c) Photographs of typical bubble solutions created at discrete values of %PEG demonstrating foaming for PEG concentrations >15%.

**3.3 Effect of PEG on the stability of MBs:**

Stability of the C3F8 filled MBs were tested by measuring the MB concentration at different time points during incubation under physiological conditions in cell medium at 37 0C, and in an open environment to allow gas exchange. Figure 6 illustrates the outcome of changing the concentration of PEG2000 on the bubble lifetime. Overall, the presence of PEG on the outside of the lipid shell led to longer bubble lifetimes compared to lipid only coated MBs. The MB lifetime was found to correlate with the total MB concentration, such that at 4 and 5% PEG where the highest concentration of MBs is produced also showed the greatest increase in MB lifetime with further increases in PEG concentration leading to slightly faster rates MB decay.



Figure 6: MB lifetime for C3F8 filled bubbles at 37°C in cell medium as a function of PEG concentration. (Lines represent best fit to an exponential decay).

The lifetime data in Figure 6 are fitted to an exponential decay and the time constant, τ (time it takes to reach 1/e (37%) of the original concentration) determined. In absence of PEG, the bubble concentration reaches 37% of the original concentration in ~13 mins, and increased to 50 mins on adding 2% PEG, compared to 55and 60 mins in the presence of 4, 5% PEG respectively in the lipid shell. The 10 and 15% PEG samples show reduced lifetimes that resembles decay time in case of 2% PEG, for the 25 and 35% PEG were close to each other reaching the same concentration in 33 and 31 mins respectively. Figure 7 shows that the MB concentration and MB lifetime are highly correlated. One can clearly see that the maximum concentration and lifetime of bubbles occur at 4-5% PEG and gradually reduces with increasing concentrations.



Figure 7: Effect of increasing the PEG concentration on bubble concentration and lifetime. The left axis presents the concentration of formed bubbles (open squares), the right axis presents the time to reach 1/e of the original bubble concentration (open circles).

**3.4 Effect of PEG on the mechanical properties of MBs:**

The mechanical properties of a MB are known to be related to the shell stiffness. In general, the shape of the deformation curve under loading is the same for all the maximum loads tested. We have determined the effective stiffness of the MBs increased as a function of maximum applied load, Figure 8, following the protocols outlined previously [[25](#_ENREF_25)]. Figure 8a shows data for MBs without any PEG coating and indicates that the lipid composition has negligible effect on the MB stiffness, over the range of compositions studied here. The effect of different PEG concentrations is shown Figure 8b.

At the lowest applied force, 5nN, all PEG concentrations give similar stiffnesses reflecting that at low forces we expect to be probing the MB local shell stiffness. At higher applied forces, the stiffness values of MBs diverged with increasing amounts of %PEG. For each PEG concentration the MB shell stiffness was found to vary linearly with applied force. The effective stiffness of the brush layer can be estimated by considering the difference between the PEGylated MB stiffness and the plain lipid shell MB in absence of PEG at a given load. Figure 9 gives the increase in the MB stiffness from a load at 5nN to 50nN for a given %PEG (i.e. [(k@50nN-k@5nN) for X% PEG]), along with the augmentation (or change) in the stiffness at 50nN load (i.e. [(k@50nN-k@5nN) for X% PEG] – [(k@50nN-k@5nN) for 0% PEG]) for all %PEG. The augmentation or change in stiffness at 50nN load shows a slight increase at zero to 10% PEG, followed by a steep linear increase from 15 to 35%, which may be related to the change in the PEG configuration from mushroom to brush at increasing PEG concentrations.



Figure 8: AFM stiffness measurements for MBs, (a) control experiment for MB with no PEG chains, MBs have the minimum and maximum% of DOPE lipid as a source for biotin binding to the surface. (b) MBs with different DSPE-PEG concentrations. Error bars represents averages of 5 bubbles for each case, all bubble sizes ranges between 3-4 µm.

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Figure 9: The Table shows the difference in MB stiffness between loads at 50nN and 5nN (k@50N-k@5N) and the increase in MB stiffness at a given %PEG compared to 0% PEG, [(k@50N-k@5N) for X% PEG] – [(k@50N-k@5N) for 0% PEG]. This increase or change in stiffness as a function of %PEG is plotted and shows a linear increase above 10% PEG, (dashed line for guidance only).

**4 Discussion and Conclusion:**

Grafting PEG has been widely used for therapeutic applications to increase hydrophilicity and increase circulation lifetime, mainly to provide stealth properties for liposomes [[16](#_ENREF_16), [32](#_ENREF_32), [33](#_ENREF_33)], nanoparticles [[4](#_ENREF_4), [5](#_ENREF_5), [15](#_ENREF_15)] and MBs [[6](#_ENREF_6), [7](#_ENREF_7), [23](#_ENREF_23)]. De Gennes[[34](#_ENREF_34)] suggested the existence of different conformational behaviour of surface grafted PEG, ranging from mushroom to brush regimes depending on the grafting density. Since then, many systematic investigations took place to confirm this dependence together with the PEG molecular weight and the layer thickness [[19](#_ENREF_19), [22](#_ENREF_22)]. In this study a range of concentrations of PEG has been used from 2% to 35% PEG coverage, i.e. whole range from mushroom, mushroom-brush border configuration, to well within the brush regime. The effect of these different PEG configurations on the MB characteristics was the main aim of this study. Incorporating PEG into the lipid shell led to increased MB concentration and consequently increased MB lifetime (presumably through a reduction in the driving force for dissolution). At higher concentrations ≥15 % PEG foaming occurred, accompanied with a reduction in the MB concentrations. Proposed reason for this is related to the high concentration and packing arrangement in the brush mode, where the repulsive forces between the PEG chains increase and cause disruption in the lipid membrane [[35](#_ENREF_35)]. It was previously mentioned that incorporating PEG in the MB shell increases the concentration and stability as a result of hindering coalescence and foaming [[36](#_ENREF_36)].

Similar behaviour has been reported in studies of bilayers and liposomes, where the incorporation of too much PEG leads to disorder in the structure of the lipid membrane and increased membrane permeability [[20](#_ENREF_20), [32](#_ENREF_32)] In studies involving X-ray diffraction analysis for osmotically stressed liposomes with PEG2000 at concentrations ranging from 0-15mol% [[19](#_ENREF_19), [37](#_ENREF_37)], it was demonstrated that increasing the PEG concentration in the mixture results in an increase in the phase separation between lipids which could be one of the reasons behind the shorter lifetime of our MBs. Another study mapped the phase transition boundaries for binary systems incorporating DSPC-PEG2000 [[38](#_ENREF_38)]. This indicated that the DPPC/DSPE-PEG2000 binary system is completely miscible for large regions of the phase space diagram (for <15-20% DSPE-PEG2000) at sufficiently high surface pressures (>15-20mN/m) which is expected for the MB shell due to the Laplace over-pressure inside. This implies that this MB composition is highly suitable as a delivery vehicle because the lipopolymer shell exists as single condensed phase with low permeability imparting good stealth properties with PEG evenly distributed on the surface. At higher DSPE-PEG2000 concentrations ~15-60% phase separation exists as two condensed DPPC and DSPE phases. The phase behaviour may be correlated to the augmentation of the shell stiffness we have found here at increasing PEG composition (Fig. 9), but to confirm this more detailed studies are required. Other studies for example have investigated the gelation behaviour of lipopolymers [[39](#_ENREF_39)], suggesting that the MB properties we observe at higher DSPE-PEG2000 concentrations could be related to 2D gelation at the air-water interface.

Transitions in MB concentration can be related to the shell composition in a two-component system and may occur when one of the components reaches its percolation threshold. For example, a DSPC/PEG40S system made using the shaking method shows a jump in MB concentration from 1x108 to 5x109 when the DSPC reaches 0.7 mol fraction (or more) [[36](#_ENREF_36)]. For our DPPC/DSPE-PEG2000 system, we have only tested bubbles in the range up to 0.35 mol fraction of the PEG-lipid, so it is possible that the MB tested here are within the percolation threshold of DPPC. To our knowledge, our study is the first to investigate MB formation and properties with such fine changes in %PEG composition and therefore the role of percolation networks in our system and more generally in lipid-shelled MBs containing PEG is still to be determined.

The effect of PEG concentration on the mechanical properties of MBs showed that at low loading forces (~5 to 10nN) one probes mainly the local MB shell stiffness whereas higher loads probe a whole MB response, with consequently more complex behaviour and interpretation. The MB stiffnesses presented here are within the same range with previously reported stiffness results by AFM compression for other lipid-shelled Definity MBs at 39mN/m [[40](#_ENREF_40)]. The results presented in Figure 8 show that the MB stiffness is similar for all bubbles at 5nN load, suggesting that the in-plane shell properties do not change significantly with increasing PEG. This idea remains to be tested more thoroughly, but would indicate that the shell stiffness is dominated by the lipid-lipid interactions compared to PEG-PEG interactions at low compression. This makes sense as the lipid-lipid interactions should drive the shell formation at the gas-liquid interface. The effective MB stiffness increases with increasing the applied load which is a behaviour that we have observed before with different lipid MB architectures [[25](#_ENREF_25)]. Comparing the results presented in Figure 8a and b indicates that the observed change in stiffness at different PEG concentrations is determined to the presence of the PEG chain, rather than a change in the lipid composition. The effective stiffness at the highest loads of 50 nN is similar for the 0, 5 and 10% PEG bubbles. For the 15% and higher concentrations the stiffness increased linearly with PEG concentration (Figure 9) due to the reduced distances between adjacent PEGs, together with increasing the repulsive force between these chains results in restricting the range of motion of the PEG chains [[5](#_ENREF_5), [35](#_ENREF_35)]. This reduced mobility will stiffen the brush layer surrounding the MB. In another AFM study on bilayers, it has also been reported that increasing the concentration of PEG has major consequences on the membrane fluidity causing loss of lipid mobility in the membrane [[41](#_ENREF_41), [42](#_ENREF_42)]. The similarity in stiffness values at low applied force (5nN) and the increasing divergence at higher forces could imply that the lipid shell surrounding the gas core deforms first, possibly followed by the compression of the rigid PEG brush layer. Previous estimations of the applied pressure in such AFM deformation experiments with a tip-less cantilever on MBs of 3-4 μm diameter, indicate that it is in the region of 103-104 Pa [[25](#_ENREF_25)]. This is a lower limit for the applied pressure and is based on a simple geometric calculation and the assumption that there is perfectly equal deformation between the top and bottom surfaces of the MB (i.e. at the cantilever interface and the supporting surface interface). Typically, however, the applied pressure increases approximately linearly with the maximum applied force, and for 50nN is ~5 x 103 Pa for the lipid+PEG2000 shelled MBs. This pressure is too low to indent the PEG brushes, based on previous experimental and theoretical work on indentation of supported PEG-lipid bilayers using an AFM tip [[42](#_ENREF_42)]. This work indicated that the pressure needs to be in the region of 105-106 Pa before the PEG chains yield. So although our conversion of applied force to pressure is based on a geometrical calculation as a lower limit, it is most likely that the PEG brushes on the outside of these MBs are not being significantly deformed and that increasing the PEG density increases the effective stiffness of the whole MB as opposed to the local bending stiffness of the shell.

In conclusion, increasing the concentration of PEG coverage on the outside of the MB shell results in changing the configuration of PEG chains around the lipid shell from mushroom to brush then dense brush configuration as previously proposed by De Gennes. The PEG concentration and configuration influence the abundance and lifetime of the bubbles produced, with the two parameters being correlated. The changes in the mechanical properties of the MB attributed to the brush configuration have been monitored with the AFM, where the effective brush stiffness increased by increasing the density of the brush layer. The results indicate that relatively low incorporation of PEG-lipid (~5% in our formulation) is required to optimise the MB abundance and lifetime while retaining similar mechanical properties, which at low loads are dominated mainly by the lipid-lipid interactions. These outcomes are important for designing new MBs for therapeutic delivery where a high MB concentration, long lifetime and evasion of the body’s immune response are required for maximum efficacy.

**5 Acknowledgment:**

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**6 References**

(1) Ferrara, K., R. Pollard, and M. Borden, Ultrasound MB contrast agents: Fundamentals and application to gene and drug delivery. *Annu Rev Biomed Eng*, **2007**. 9: p. 415-47.

(2) Klibanov, A.L., MB contrast agents: Targeted ultrasound imaging and ultrasound-assisted drug-delivery applications. *Invest Radiol*, **2006**. 41(3): p. 354-62.

(3) Stride, E. and N. Saffari, MB ultrasound contrast agents: A review. *Proc Inst Mech Eng H*, **2003**. 217(6): p. 429-47.

(4) Neoh, K.G. and E.T. Kang, Functionalization of inorganic nanoparticles with polymers for stealth biomedical applications. *Polymer Chemistry*, **2011**. 2(4): p. 747-759.

(5) Owens, D.E. and N.A. Peppas, Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *International Journal of Pharmaceutics*, **2006**. 307(1): p. 93-102.

(6) Chen, C.C. and M.A. Borden, Ligand conjugation to bimodal poly(ethylene glycol) brush layers on MBs. *Langmuir*, **2010**. 26(16): p. 13183-13194.

(7) Lozano, M.M. and M.L. Longo, MBs coated with disaturated lipids and dspe-peg2000: Phase behavior, collapse transitions, and permeability. *Langmuir*, **2009**. 25(6): p. 3705-3712.

(8) Borden, M.A. and M.L. Longo, Dissolution behavior of lipid monolayer-coated, air-filled MBs: Effect of lipid hydrophobic chain length. *Langmuir*, **2002**. 18(24): p. 9225-9233.

(9) Ferrara, K.W., M.A. Borden, and H. Zhang, Lipid-shelled vehicles: Engineering for ultrasound molecular imaging and drug delivery. *Acc Chem Res*, **2009**. 42(7): p. 881-92.

(10) Hashizaki, K., H. Taguchi, C. Itoh, H. Sakai, M. Abe, Y. Saito, and N. Ogawa, Effects of poly(ethylene glycol) (peg) chain length of peg-lipid on the permeability of liposomal bilayer membranes. *Chem Pharm Bull (Tokyo)*, **2003**. 51(7): p. 815-20.

(11) Hashizaki, K., H. Taguchi, K. Tsuchiya, H. Sakai, M. Abe, Y. Saito, and N. Ogawa, Calorimetry and cryo-transmission electron microscopic studies of peg2000-grafted liposomes. *Chem Pharm Bull (Tokyo)*, **2006**. 54(4): p. 561-3.

(12) Hashizaki, K., H. Taguchi, C. Itoh, H. Sakai, M. Abe, Y. Saito, and N. Ogawa, Effects of poly(ethylene glycol) (peg) concentration on the permeability of peg-grafted liposomes. *Chem Pharm Bull (Tokyo)*, **2005**. 53(1): p. 27-31.

(13) Garbuzenko, O., Y. Barenholz, and A. Priev, Effect of grafted peg on liposome size and on compressibility and packing of lipid bilayer. *Chemistry and Physics of Lipids*, **2005**. 135(2): p. 117-129.

(14) Silvander, M., M. Johnsson, and K. Edwards, Effects of peg-lipids on permeability of phosphatidylcholine/cholesterol liposomes in buffer and in human serum. *Chemistry and Physics of Lipids*, **1998**. 97(1): p. 15-26.

(15) Perry, J.L., K.G. Reuter, M.P. Kai, K.P. Herlihy, S.W. Jones, J.C. Luft, M. Napier, J.E. Bear, and J.M. DeSimone, Pegylated print nanoparticles: The impact of peg density on protein binding, macrophage association, biodistribution, and pharmacokinetics. *Nano Letters*, **2012**. 12(10): p. 5304-5310.

(16) Nicholas, A.R., M.J. Scott, N.I. Kennedy, and M.N. Jones, Effect of grafted polyethylene glycol (peg) on the size, encapsulation efficiency and permeability of vesicles. *Biochimica Et Biophysica Acta-Biomembranes*, **2000**. 1463(1): p. 167-178.

(17) Kupiainen, M., E. Falck, S. Ollila, P. Niemela, A.A. Gurtovenko, M.T. Hyvonen, M. Patra, M. Karttunen, and I. Vattulainen, Free volume properties of sphingomyelin, dmpc, dppc, and plpc bilayers. *Journal of Computational and Theoretical Nanoscience*, **2005**. 2(3): p. 401-413.

(18) Petrache, H.I., S.W. Dodd, and M.F. Brown, Area per lipid and acyl length distributions in fluid phosphatidylcholines determined by h-2 nmr spectroscopy. *Biophysical Journal*, **2000**. 79(6): p. 3172-3192.

(19) Kenworthy, A.K., K. Hristova, D. Needham, and T.J. McIntosh, Range and magnitude of the steric pressure between bilayers containing phospholipids with covalently attached poly(ethylene glycol). *Biophysical Journal*, **1995**. 68(5): p. 1921-1936.

(20) Li, S.D. and L. Huang, Nanoparticles evading the reticuloendothelial system: Role of the supported bilayer. *Biochim Biophys Acta*, **2009**. 1788(10): p. 2259-66.

(21) Degennes, P.G., Polymers at an interface - a simplified view. *Advances in Colloid and Interface Science*, **1987**. 27(3-4): p. 189-209.

(22) Damodaran, V.B., C.J. Fee, T. Ruckh, and K.C. Popat, Conformational studies of covalently grafted poly(ethylene glycol) on modified solid matrices using x-ray photoelectron spectroscopy. *Langmuir*, **2010**. 26(10): p. 7299-7306.

(23) Chen, C.C. and M.A. Borden, The role of poly(ethylene glycol) brush architecture in complement activation on targeted MB surfaces. *Biomaterials*, **2011**. 32(27): p. 6579-6587.

(24) Wrenn, S.P., M. Mleczko, and G. Schmitz, Phospholipid-stabilized MBs: Influence of shell chemistry on cavitation threshold and binding to giant uni-lamellar vesicles. *Applied Acoustics*, **2009**. 70(10): p. 1313-1322.

(25) Abou-Saleh, R.H., S.A. Peyman, K. Critchley, S.D. Evans, and N.H. Thomson, Nanomechanics of lipid encapsulated MBs with functional coatings. *Langmuir*, **2013**. 29(12): p. 4096-103.

(26) Glynos, E., V. Sboros, and V. Koutsos, Polymeric thin shells: Measurement of elastic properties at the nanometer scale using atomic force microscopy. *Materials Science and Engineering B-Advanced Functional Solid-State Materials*, **2009**. 165(3): p. 231-234.

(27) Sboros, V., E. Glynos, S.D. Pye, C.M. Moran, M. Butler, J.A. Ross, W.N. McDicken, and V. Koutsos, Nanomechanical probing of MBs using the atomic force microscope. *Ultrasonics*, **2007**. 46(4): p. 349-54.

(28) Peyman, S.A., R.H. Abou-Saleh, J.R. McLaughlan, N. Ingram, B.R. Johnson, K. Critchley, S. Freear, J.A. Evans, A.F. Markham, P.L. Coletta, and S.D. Evans, Expanding 3d geometry for enhanced on-chip MB production and single step formation of liposome modified MBs. *Lab Chip*, **2012**. 12(21): p. 4544-52.

(29) Heath, G.R., R.H. Abou-Saleh, S.A. Peyman, B.R.G. Johnson, S.D. Connell, and S.D. Evans, *Self-assembly of actin scaffolds on lipid MBs*, in *Soft Matter*. 2014.

(30) Jeuken, L.J.C., N.N. Daskalakis, X.J. Han, K. Sheikh, A. Erbe, R.J. Bushby, and S.D. Evans, Phase separation in mixed self-assembled monolayers and its effect on biomimetic membranes. *Sensors and Actuators B-Chemical*, **2007**. 124(2): p. 501-509.

(31) Hutter, J.L. and J. Bechhoefer, Calibration of atomic-force microscope tips (vol 64, pg 1868, 1993). *Review of Scientific Instruments*, **1993**. 64(11): p. 3342-3342.

(32) Dos Santos, N., C. Allen, A.M. Doppen, M. Anantha, K.A.K. Cox, R.C. Gallagher, G. Karlsson, K. Edwards, G. Kenner, L. Samuels, M.S. Webb, and M.B. Bally, Influence of poly(ethylene glycol) grafting density and polymer length on liposomes: Relating plasma circulation lifetimes to protein binding. *Biochimica Et Biophysica Acta-Biomembranes*, **2007**. 1768(6): p. 1367-1377.

(33) Immordino, M.L., F. Dosio, and L. Cattel, Stealth liposomes: Review of the basic science, rationale, and clinical applications, existing and potential. *International Journal of Nanomedicine*, **2006**. 1(3): p. 297-315.

(34) Degennes, J.L., Lipids profile in 1987 - where to start, and where to end. *Annales De Cardiologie Et D Angeiologie*, **1987**. 36(10): p. 561-565.

(35) Li, S.D. and L. Huang, Nanoparticles evading the reticuloendothelial system: Role of the supported bilayer. *Biochimica Et Biophysica Acta-Biomembranes*, **2009**. 1788(10): p. 2259-2266.

(36) Kwan, J.J. and M.A. Borden, Lipid monolayer collapse and MB stability. *Advances in Colloid and Interface Science*, **2012**. 183: p. 82-99.

(37) Kenworthy, A.K., S.A. Simon, and T.J. McIntosh, Structure and phase-behavior of lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol). *Biophysical Journal*, **1995**. 68(5): p. 1903-1920.

(38) Lozano, M.M. and M.L. Longo, Complex formation and other phase transformations mapped in saturated phosphatidylcholine/dspe-peg2000 monolayers. *Soft Matter*, **2009**. 5(9): p. 1822-1834.

(39) Foreman, M.B., J.P. Coffman, M.J. Murica, S. Cesana, R. Jordan, G.S. Smith, and C.A. Naumann, Gelation of amphiphilic lipopolymers at the air−water interface:  2d analogue to 3d gelation of colloidal systems with grafted polymer chains. *Langmuir*, **2003**. 19(2): p. 326-332.

(40) Santos, E.B., J.K. Morris, E. Glynos, V. Sboros, and V. Koutsos, Nanomechanical properties of phospholipid MBs. *Langmuir*, **2012**. 28(13): p. 5753-60.

(41) Albertorio, F., A.J. Diaz, T.L. Yang, V.A. Chapa, S. Kataoka, E.T. Castellana, and P.S. Cremer, Fluid and air-stable lipopolymer membranes for biosensor applications. *Langmuir*, **2005**. 21(16): p. 7476-7482.

(42) Kaufmann, S., O. Borisov, M. Textor, and E. Reimhult, Mechanical properties of mushroom and brush poly(ethylene glycol)-phospholipid membranes. *Soft Matter*, **2011**. 7(19): p. 9267-9275.