Light-sensitive fusion between polymer-coated liposomes following physical anchoring of polymerisable polymers onto lipid bilayers by self-assembly

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Delivery of therapeutics (drugs, radionuclides or genes) *in vivo* can be optimized when carried by a targeting delivery vehicle such as a surfactant vesicle, polymeric micelle or other polymer-coated colloidal particulate. In the present communication, we propose a general method based on self-assembly principles, to construct lipid-polymer bilayer vesicles whose featured characteristics may be altered according to the polymer molecule used, thus be easily designed along the needs of a particular delivery application. Polymer molecules containing non-polymerizable (polypropylene) and polymerisable (methacrylate) hydrophobic groups were used to construct lipid-polymer vesicles by following two different methods of preparation. In accord with our previous findings, when both types of polymer molecules are added to pre-formed liposomes, only weak adsorption onto the lipid surface occurs. Preparation of the vesicles by pre-mixing the lipid and polymer molecules has proved essential in order to allow the hydrophobic blocks of the copolymers to participate as integral parts of the bilayer. Anchoring of a polymerisable polymer onto the lipid bilayer by hydrophobic interactions, resulted in steric stabilization of the vesicles. When UV polymerization of the bilayer-incorporated [Methyl(PEG)$_{2000}$MA] polymer was induced, inter-vesicle fusion was triggered. Direct cryo-EM imaging of fusion between the PEG-coated liposomes has been observed. Such sterically stabilised fusogenic vesicles were constructed as potential triggered-release delivery systems, responsive to a variety of external stimuli depending on the type of polymerisable, hydrophobic group in the polymer molecule. By altering the properties of the incorporated hydrophobic group, liposomes able to fuse in response to initiators milder than UV light, such as green or red light, sound, temperature, oxygen or pH can be engineered.

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Introduction

Engineering of liposome surfaces and bilayers is primarily aimed at improving the delivery of transported molecules and their controlled release onto targeted sites (*in vitro* or *in vivo*), for therapeutic or other purposes. There have been two major directions in liposome engineering research: (a) control of the rigidity and physicochemical stability of the liposome bilayer; and (b) rendering liposome surfaces recognisable by specific receptors at the target sites. Strategies whereby modification of the liposome surface is sought have been playing a leading role in the development of clinically relevant liposome systems during the last 15 years.[1,2] Placing an effective barrier (most commonly a neutral, hydrophilic macromolecular chain) protruding from the liposome surface to the external aqueous environment contributed to the rejuvenation of interest in the liposome field and led to an extremely rapid approval of liposome-based pharmaceuticals, clinically used in chemotherapeutic applications today.[3] Irrespective of the way in which the macromolecules are attached onto the liposome surface, the mechanism by which these systems function *in vitro* and *in vivo* is by steric stabilisation.[4,5] The term "Stealth"[6] has been coined to describe these systems, originating from their proposed *in vivo* behaviour leading to disguise from the blood serum macrophages and consequently, delayed recognition and sequestration by the RES (mainly the mononuclear phagocytic cells of the liver and spleen).[7,8]

Most of the ways to alter liposome surface characteristics in order to form sterically stabilized systems involve chemical conjugation of a polymer chain to the headgroup of a particular lipid (usually PE). An alternative methodology we developed almost a decade ago is aimed at engineering sterically stabilized liposomes by application of the concept of 'physical grafting'.[9–11] 'Physical grafting' constitutes one of the principal ways by which amphipathic proteins interact with the lipid bilayers to form plasma membranes. By mimicking this well documented grafting mechanism, abundantly found in nature, novel sterically stabilized liposomes were engineered by use of synthetic amphiphilic macromolecules.[9,12] Application of the 'physical grafting' method, eliminates the need for covalent chemical manipulation of the liposome-participating lipids, and can also be used as a generic strategy to engineer liposome surfaces by any macromolecule (synthetic or biological), provided its molecular structure allows for bilayer incorporation, therefore an effective physical anchor. However, the presence of polymeric macromolecules (chemically or physically grafted) at the liposome surfaces, pharmacologically leads only to prolonged blood circulation half-lives without incurring any means of controlling the release of encapsulated material at specific sites in the body. Even though the polymer-coated, steric stabilisation of liposomes is offering enhanced accumulation in the case of tissues exhibiting 'leaky' and 'faulty' vasculature (tumors, sites of infection) by a mechanism called 'passive targeting',[13] control of load release is still highly desirable. Inclusion of triggerable release functionalities has been attempted in the design of numerous liposome systems, particularly taking advantage of moderate temperature increases[14,15] and pH fluctuations.[16]

In the present study we extended application of 'physical grafting' of block copolymers onto lipid bilayers to include a polymerisable polymer containing a polymerisable hydrophobic group. Construction of photo-triggerably fusogenic sterically stabilised liposomes was therefore achieved, by incorporation of the polymer within the lipid bilayer by self-assembly, able to act both as the steric barrier (PEG domain) and the triggerable molecular switch (methacrylate domain) for interliposomal fusion.

Materials and methods

Liposome preparation and polymerisation

All liposomes were prepared using soybean lecithin (Sigma, UK) and doubly-distilled water. The tri-block copolymer, PF127 (PEO_{99}-PPO_{65}-PEO_{99}) was a gift from ICI Surfactants, Belgium and was used without further purification. The polymerizable polymer: methoxy-poly(ethylene glycol)-methacrylate [CH_3-O$(PEG)_{2000}$COCO(CH_3)] (M-$(PEG)_{2000}$MA); a gift from ICI Paints, Slough, UK) was used without further purification and was included in the lipid mixture following liposome preparation or during liposome formation. Azobisisobutyronitrile, AIBN (BDH, UK), was also included in the initial solvent lipid and polymer mixtures. This mixture of lipid, polymer and AIBN

was dissolved in a chloroform : methanol (2 : 1) solvent phase, which was then rotary evaporated. A thin lipid or lipid-polymer film was rehydrated to form multilamellar vesicles (MLVs) and subsequently sonicated using a 50 Hz bath sonicator (Kerry) under nitrogen atmosphere and constant temperature (25 °C). Liposomes were filtered through Millipore 0.2 μm polycarbonate filters following sonication. A polymerization reaction was photochemically initiated using a tailor-made double jacketed quartz vessel with circulating water (Syngenta plc., formerly ICI Agrochemicals, Berkshire, UK), achieving UV illumination (125 W mercury lamp used as source of ultraviolet radiation; max output 240 nm light wavelength) of the sterically stabilized SUVs containing AIBN, by continuous stirring of the sample at constant temperature. The vesicle solutions were photo-irradiated while stirred constantly at 25 °C.

^{1}H-NMR

All spectra were recorded on a JEOL GX400 spectrometer, acquired with 45′ pulses and a 2 s repetition rate. All samples were prepared in D_2O (Aldrich, UK) at a fixed lipid concentration of 2% (w/w).

Optical microscopy

A Leitz Diaplan light microscope was used connected to a Cohu CCD camera. All optical microscopy images were obtained using a 40× objective lens under phase contrast.

Cryogenic transmission electron microscopy

Vesicles were imaged using a Zeiss EM 902 cryo-transmission electron microscope exactly as described elsewhere.[17] Briefly, samples were prepared using a custom-built controlled environment vitrification system, allowing immersion of the copper or carbon grids used to deposit a drop of the sample, into liquid ethane under controlled temperature and humidity. The accelerating voltage was 80 kV and the cryo-stage temperature was kept below 180 K.

Quasielastic light scattering

All measurements were carried out using Malvern 4700 (Malvern, UK) at a fixed 90° angle and 25 °C. The instrument cumulants analysis software was used for the mean diameter measurements. At least three independent series of ten measurements were carried out. The experimental error from the sizing measurements was never above 1% of the mean value.

Laser Doppler electrophoresis

The electrophoretic mobility of the liposomes was measured using the DELSA 440 Zetasizer instrument (Beckman-Coulter, UK) operating with a helium-neon laser beam (5 mW). Ten different measurements for each liposome system were carried out and all four different angles of detection were used to obtain the ζ-potential at the liposome surface by employing the Smoluchowski approximation on the electrophoretic mobility data obtained when a 5 V electric field was applied to the liposome suspension.

Results

In agreement with our previous findings, we observed that interaction of both the tri-block copolymer or polymerisable polymer molecules with the lipid bilayer by purely hydrophobic forces, resulted in polymer coating (steric stabilization) of the vesicles. This was done by preparation of liposomes containing PF127 and M-$(PEG)_{2000}$MA with the initial lipid mix in solvent, or addition of polymer molecules post-liposome formation. Fig. 1 shows the ^{1}H-NMR spectrum for a small unilamellar liposome dispersion (2% w/w) without addition of any polymer (A), following addition of 1% w/w PF127 molecules in the solvent mix (B) and after liposome preparation (C). Line broadening of the methyl peak in the hydrophobic PPO block was observed (black arrow), illustrating restriction of the PPO molecular movement when incorporated in the lipid bilayer

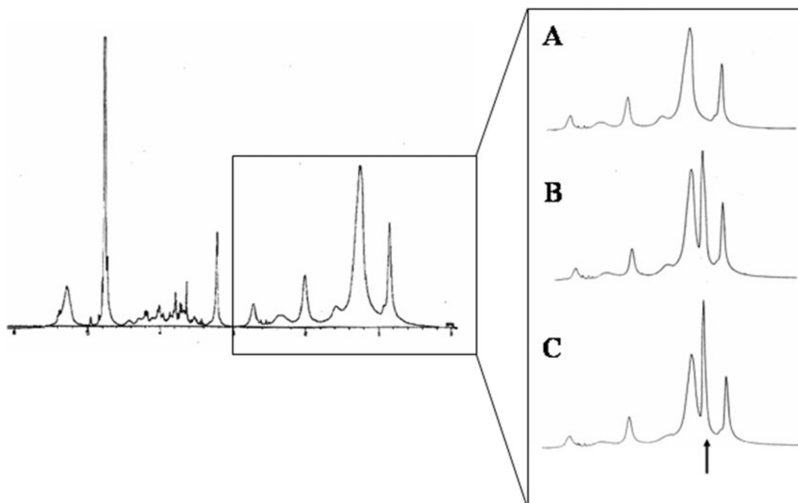

Fig. 1 ^{1}H-NMR spectrum of small unilamellar vesicles, and expansions of the spectral regions where the methyl signal from the PPO groups of the tri-block copolymer PF127 appears. (A) liposome alone; (B) liposome with PF127 mixed with the lipid molecules in solvent, prior to lipid hydration; (C) liposome with PF127 added post liposome formation.

(B). The structural characteristics of those multilamellar and unilamellar vesicles incorporating the copolymer were then studied by optical videomicroscopy and cryo-EM (Fig. 2). Interestingly, when PF127 molecules (1% w/w) were allowed to participate during liposome formation, structural changes occurred for both the MLV and SUV. Participation of the PPO blocks into the lipid bilayer led to elongation of the spherical MLVs (Fig. 2A) to rod-like structures (Fig. 2B - black arrows). The cryo-EM examination of the same samples following the high energy sonication process to

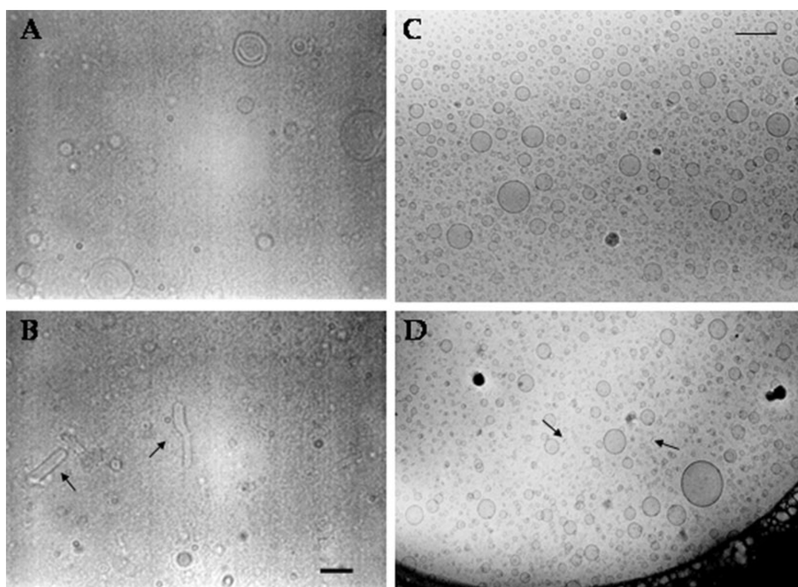

Fig. 2 Optical (A and B) and Cryo-EM (C and D) images of multilamellar (MLV) and small unilamellar (SUV) vesicles. (A) MLV alone; (B) MLV with PF127 added prior to lipid hydration; (C) SUV alone; (D) SUV with PF127 added prior to lipid hydration. Black arrows indicate the elongated structures formed in the presence of PF127. Scale bar for A and B is 1 μm; for C and D is 100 nm.

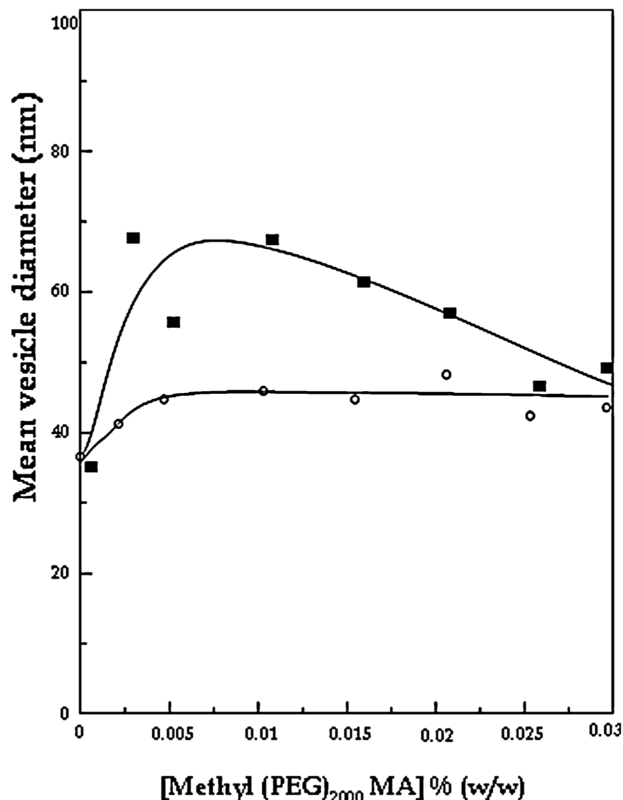

Fig. 3 Mean vesicle diameter by quasielastic light scattering for increasing $M(PEG)_{2000}MA$ concentrations. Filled squares are vesicles formed in the presence of polymer; open circles are vesicles with polymer added post-liposome formation.

form the SUVs, was indicative of elongated small flake-like structures only when F127 was allowed to participate in liposome formation at this molar ratio (approximately one copolymer molecule for every 30 lipids in the bilayer; Fig. 2D - black arrows). Both microscopic techniques indicated that incorporation of the tri-block copolymer molecule into the lipid bilayer was leading to structural alterations in the resultant lipid-polymer vesicles.

Fig. 3 shows QUELS mean particle diameter values following interaction between the $M\text{-}(PEG)_{2000}MA$ and lipids following the two different protocols described above for the tri-block copolymer. In the case of mixing the polymerisable polymer and the lipids in the chloform:methanol solvent followed by evaporation and hydration, the resultant SUV mean diameter peaked at a range of lipid:copolymer molar ratio. On the other hand, addition of the polymer molecules following liposome preparation led to vesicle surface adsorption evidenced by a moderate increase in the mean particle diameters. The molar ratio range between 6 : 8 lipids per polymer molecule was found to lead to maximum increases in mean vesicle size. Above this molar ratio, decreases in the mean particle diameter for the vesicles containing bilayer-anchored polymers are thought to be indicative of gradual lipid bilayer solubilisation towards the more preferred mixed micellar phase, with co-partitioning of the vesicle and mixed micelles in-between.

The effect on vesicle surface charge following the two types of interaction between the $M\text{-}(PEG)_{2000}MA$ and lipids were monitored by studying the ζ-potential at the vesicle surface (Fig. 4). Decreases in the ζ-potential of the liposome surface from -51.3 mV for the 'naked' liposomes to values between -30 and -40 mV for vesicles resulting from both types of polymer interaction with the lipids, indicates the presence of the hydrophilic polymer PEG layer at the liposome surface. The presence of polymer both prior and post liposome formation was therefore leading to polymer-coated, sterically stabilised vesicles.

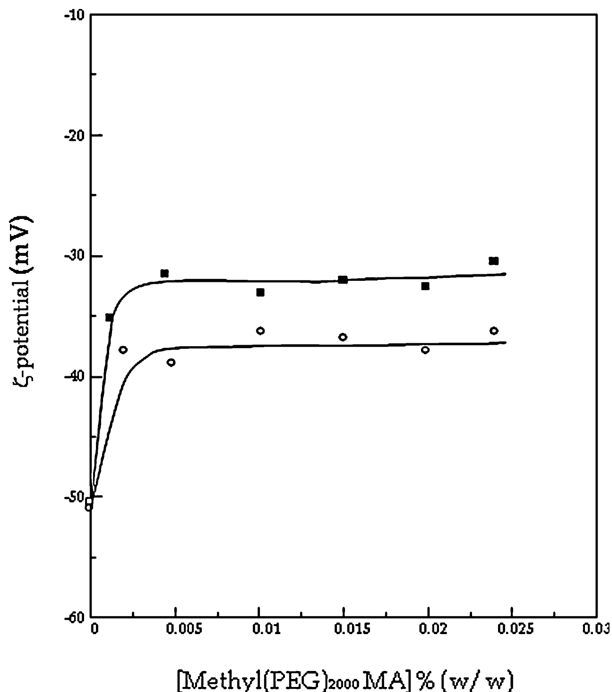

Fig. 4 Vesicle surface charge (ζ-potential) increasing $M(PEG)_{2000}MA$ concentrations. Filled squares are vesicles formed in the presence of polymer; open circles are vesicles with polymer added after liposome formation.

Vesicles (2% lipid w/w) that contained 'physically grafted' polymerisable polymer (*i.e.* bilayer incorporated) and the hydrophobic polymerisation initiator AIBN in the bilayer, were subsequently placed in a quartz polymerisation vessel and irradiated with UV light for different time points at 25 °C. Fig. 5 shows the cryo-EM microscopy of $M\text{-}(PEG)_{2000}MA$ incorporating vesicles before (Fig. 5A) and after 12 h of UV light irradiation (Fig. 5B). Inter-vesicle fusion leading to significantly larger closed vesicular structures can be observed. Determination of mean vesicle diameter for these vesicle systems by QUELS did not provide meaningful data because of the large, non-spherical, inhomogenous structures formed. Interestingly, five times dilution of this polymerised vesicle dispersion with double distilled water (Fig. 5C), led to rupture of the large vesicular structures providing an SUV dispersion of approximately the same mean vesicle size as the original sterically stabilised formulation prior to polymerisation (Fig. 5A). These images were indicative to us that vesicle fusion was triggered when a photochemical polymerization reaction between the hydrophobic blocks of the bilayer-buried polymer (methacrylate groups) was induced by UV light irradiation.

Discussion

In the present study we applied the principle of 'physically grafting' tri-block copolymer and polymerisable polymer molecules onto lipid bilayers to obtain polymer-coated liposomes. Extension of the 'physical grafting' principle was attempted by use of polymer molecules containing a polymerisable (methacrylate) group. On photochemical initiation, polymerisation of the methacrylate predictably residing within the bilayer led to vesicle destabilisation and extensive vesicle fusion, imaged directly by cryo-EM. The fused vesicle structures resembled cryo-EM images of recently reported intra-liposomal fusion following interaction with SNARE proteins.[18]

The self-assembly of ABA block copolymers with lipid molecules during the formation of vesicle bilayers has been proposed as an alternative novel method to sterically stabilize phospholipid

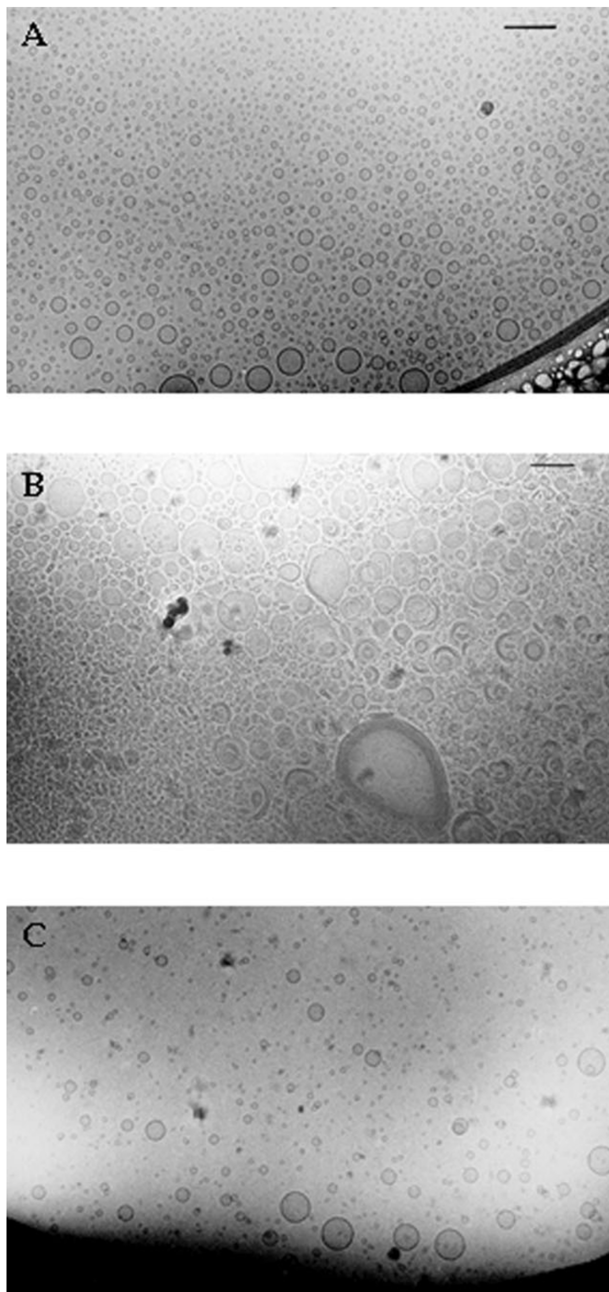

Fig. 5 Cryo-electron microscopy images of lipid:M(PEG)$_{2000}$MA vesicles formed in the presence of polymer. (A) lipid-M(PEG)$_{2000}$MA vesicles before polymerisation; (B) lipid:M(PEG)$_{2000}$MA vesicles following 12 h in the polymerisation quartz vessel; (C) sample shown in (B) diluted ×5 with double distilled water.

bilayer vesicles.[19] By allowing the anchoring B block of the copolymer to participate in the vesicle formation process a novel structure was obtained. The B block is physically grafted inside the bilayer, thus firmly anchoring the two A stabilizing chains dangling in solution. From the various experimental methods used previously[19,20] and in this study (Figs. 1–4) to elucidate the effect that the polymer molecules had on the vesicle systems, it was demonstrated that the polymer:lipid molar

ratio is an extremely important factor determining aggregate structure. Interaction between polymer molecules and lipids in a vesicular form takes place at low to moderate molar ratios. For higher polymer:lipid ratios destruction of the vesicle structures occurs, with a concomitant formation of mixed micelles between the polymers and the lipids, similar to the phase behaviour reported for the poly(ethyleneglycol)-phosphatidylethanolamine (PEG-PE) conjugates.[21] In the present studies, both vesicle structural and morphological studies in the case of tri-block copolymers (PF127; Figs. 1 and 2) and vesicle particle size and surface characterization for the polymerisable polymer (M-$(PEG)_{2000}$MA; Figs. 3 and 4) revealed that 'physical grafting' of the hydrophobic group of the polymers and vesicle surface coating offered by the hydrophilic (PEO and PEG) chains was taking place.

The pronounced advantage of allowing association of the hydrophobic polymer groups with the lipid bilayer are evident, since the steric barriers are much more robust and less susceptible to desorption (caused by interparticle collisions, dilution effects, interactions with components of the biological milieu), therefore offering robust polymer coating to the vesicle. Interestingly, the concept of 'physically grafting' polymer molecules onto liposomes was subsequently reproduced independently by other groups.[22–24] More recently, in a complimentary development to our work, the thermosensitive properties of the liposomes incorporating the block copolymer molecule PF127 were explored in a series of reports.[24,25] The same group reported the enhanced *in vitro* and *in vivo* delivery of a hydrophilic dye molecule encapsulated in the internal aqueous phase of the block copolymer-incorporated liposomes using a local mild hyperthermia protocol on a subcutaneous tumor model.[26]

In an attempt to extend the concept and methodologies of 'physically grafting' macromolecules onto liposomes, the present study used a polymerisable polymer molecule containing a methacrylate (polymerizable) group as the hydrophobic domain and a methoxy-PEG group as its hydrophilic chain. By initiation of a polymerization reaction, a means for triggering intra-bilayer fusion was achieved (Fig. 5). The liposome structures were constructed in a way to combine small size (less than 100 nm; Fig. 3), surface polymer coating (*i.e.* steric stabilization; Fig. 4), and capability of inducible fusion (Fig. 5). A model mechanism describing the observed fusion between liposomes is proposed and schematically shown in Fig. 6. The methacrylate groups inside the lipid bilayer on initiation of the polymerisation reaction are thought to induce strain against the high bending curvature of the lipid bilayer towards more elongated structures as can be observed in Fig. 5B. As the reaction

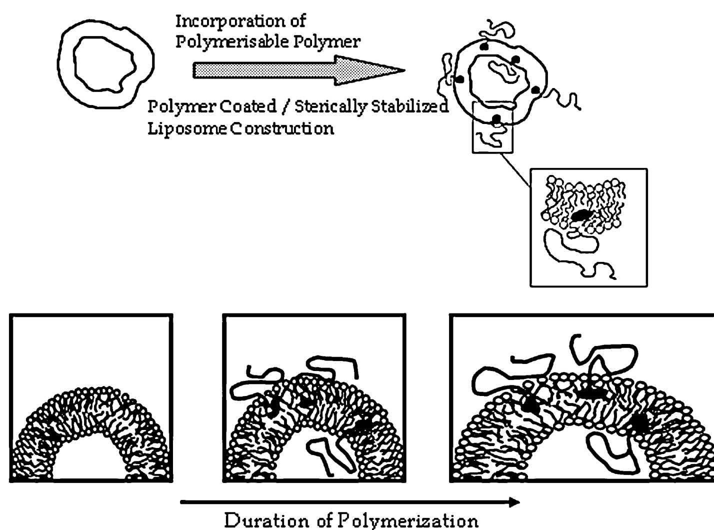

Fig. 6 Schematic representation of the 'physical anchoring' of $M(PEG)_{2000}MA$ polymer molecules into the lipid bilayer and a proposed scheme leading to intra-liposomal fusion on polymerisation.

proceeds, lateral phase separation at specific regions of the bilayer in combination with the high degree of Brownian motion (nanometer-range vesicles) leading to frequent close approach or collision between the lipid bilayers, results in fusion between the vesicles. Bilayer rigidity and lateral phase separation have been recognised as important parameters in the mechanism leading to content leakage from photopolymerised liposomes made of photochromic phospholipids,[27] however no fusion was observed or implicated in those studies.

The results obtained here also correlate well with the studies (rapid release of fluorescent marker) recently reported by O'Brien's group, by photo-inducing destabilization of sterically stabilized liposomes.[28,29] However, these investigators used newly synthesized polymerizable lipids (Bis SorbPC and analogues) and their pegylated forms instead of polymerizable polymers able to be buried within the liposome bilayer by self-assembly used in this work. Also, the perceived mechanism of bilayer destabilisation and consequent release of entrapped material from the Bis-SorbPC containing liposomes, is not through inter-vesicular fusion but due to shrinkage of the polymerised domains in the bilayer, leading to gaps through which materials release.[29] Various other triggerably fusogenic liposome systems have been engineered to release contents by acidic,[16,30] enzymatic,[31] thermal[14,15] or photochemical[32] triggering processes. Most commonly, novel DOPE analogues are synthesized, whereby external stimuli-triggered cleavage of a steric group covalently attached to the DOPE headgroup (acid-sensitivity most widely explored) exposes the cone-shaped lipid molecule, which tends to promote the bilayer-to-hexagonal phase transition facilitating membrane fusion.[33,34] Most relevant to the 'physically grafted' polymerisable polymer work we carried out herein, is the thermosensitive activation of liposomes by incorporation of *N*-isopropylacrylamide (NIPAM) polymers[12] and copolymers.[35,36] However, the inclusion of an effective steric stabilization barrier onto the NIPAM-thermosensitive liposomes without jeopardizing the temperature-responsive fusion and content release requires further work as recently described.[37] Moreover, in those studies the mechanism of thermosensitive content release has not drawn upon occurrence for inter-vesicle fusion, nor such fusion as evidenced in our studies has been previously observed. We herein propose a general method to engineer triggerably fusogenic, sterically stabilized vesicles that can be developed further by the self assembly between lipid and copolymer molecules responsive to other stimuli, as for example changes in oxidation,[38] constituting the next generation, of 'smarter' polymer–lipid hybrid vesicle systems.

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