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Abstract

The composition of microcapsules designed for drug delivery significantly impacts their properties. Ultrasound contrast agents, consisting of stabilized microbubbles (MBs), have emerged as versatile potential drug delivery vehicles to both image and overcome challenges associated with systemic chemotherapy. In our development of polylactic acid MBs decorated with immune-shielding polyethylene glycol chains, we have shown that the balance between acoustic behavior and immune avoidance was scalable and amenable to two distinct PEGylation methods, either incorporation of 5 wt% PEGylated PLA or insertion of 1 wt% PEGylated lipid (LipidPEG) in the polymeric shell. Here we describe the effects of shell compositions on MB functionalization for use in targeted cancer therapy. We chose tumor necrosis factor-related apoptosis inducing ligand (TRAIL) as the targeting ligand, motivated by the ability to both target cells and selectively induce tumor cell death upon binding. Additionally, the MBs were designed to co-encapsulate the chemotherapeutic doxorubicin (Dox) within the shell that works with TRAIL to sensitize resistant cells. We have previously shown that the MBs shatter in response to ultrasound focused at the tumor site, delivering drug-eluting fragments. This study demonstrates the effect of shell characteristics and MB functionalization (TRAIL-ligated and Dox-loaded MBs) on the acoustic response of MBs, and the cumulative effect of shell type.

Keywords

Microbubble, shell parameters, ultrasound, targeted drug delivery, TRAIL

1. Introduction

According to the National Cancer Institute, new cases of cancer in the USA in 2016 were estimated at 1,685,210, and 595,690 people were predicted suffer fatality.[1,2] Cancer, as a whole, represents a complex class of diverse diseases with a wide variety of biological structures, treatment responsivity, and developmental processes.[3] We are seeking to facilitate minimally invasive, efficient, image-guided delivery of drugs to solid tumors using ultrasound (US). To achieve this, we are developing a drug-loaded imaging platform based on injectable US contrast agents consisting of polylactic acid (PLA) microbubbles (MBs).[4-7] We have shown that shell composition, specifically introduction of immune-shielding polyethylene glycol (PEG) groups, has significant influence over the MB acoustic properties.[6] We investigated two PEGylation techniques; addition of a PEG-PLA copolymer to the polymer-rich oil phase of a water in oil in water (w/o/w) emulsion, and as the second technique, incorporation of a PEG lipid (LipidPEG) at the same stage. Acoustic properties measured in an *in vitro* acoustic testing setup were compared with the native, unPEGylated agent. We found that loss of acoustic enhancement as measured by dB returned to the transducer with respect to MB dose occurred in a dose-dependent manner for both types of PEGylated agents (loss of signal occurred at incorporation of >5 wt% PEG-PLA and incorporation of >1 wt% LipidPEG). Importantly, immune activation was reduced, also in a dose dependent manner for the PEG-PLA agents. We concluded that the balance between acoustic behavior and improved immune avoidance was scalable and dependent on shell composition. The most productive results were obtained using PEG-PLA at 5 wt% and LipidPEG at 1 wt%.

We now investigate the effects that addition of a drug, doxorubicin (Dox), and an apoptosis-inducing targeting ligand, tumor necrosis factor-related apoptosis inducing ligand (TRAIL), have on these MB acoustic properties and the impact that shell type has on drug loading and ability to kill both TRAIL-sensitive and TRAIL-resistant cell lines. The advantage of TRAIL is that once attached to the cell surface receptors, it induces tumor cell death.[8-11] Only tumor cells exhibit the

cell surface receptors DR4 and DR5, binding to which initiates transmembrane apoptosis signaling. On the other hand, healthy cells are unaffected because the decoy receptors (DcR1 and DcR2) that exhibit on their surfaces do not process the apoptotic signal; however, binding does reduce the systemic bioavailability. Our approach would avoid this reduction in bioavailability. A further major advantage of our approach is that we have shown that the MB shell can accommodate a drug, which will facilitate a broadening of potential targets.[4,5,12-15] It has also been observed that certain cancer cells are resistant to TRAIL-induced apoptosis, limiting this approach towards a range of tumors.[16-18] Several studies have investigated methods of overcoming this resistance, identifying compounds such as proteasome inhibitors and drugs, including Dox, that can potentiate the apoptotic activity of TRAIL.[16,17,19,20] It follows that the demonstrated ability of our MBs to house Dox in the shell can be combined with TRAIL ligation to sensitize resistant cells and tumors. Co-encapsulation of a bioactive molecule is not limited to Dox, however, as a variety of hydrophilic and hydrophobic agents could be incorporated into the polymeric shell to treat a wide variety of cancer types.[4]

The potential for this system to deliver both TRAIL and Dox directly to a tumor site, preventing unproductive binding that reduces bioavailability, and reducing systemic Dox toxicity by protecting the circulation from the Dox until it is at the tumor site, is enhanced by our finding that once in the US beam, our agents undergo rapid inertial cavitation, rupture, and produce fragments in the nano range (n-Sh), capable of escape through the leaky tumor angiogenic vessels.[4,6,12-15] In this paper, we investigate the effects of our three different shell compositions, 100% PLA, 5 wt% PEG-PLA and 1 wt% LipidPEG after TRAIL ligation, Dox incorporation, and a combination of the two treatments.

2. Materials and Methods

2.1 MB Preparation and PEGylation

MBs were prepared by modifying the water/oil/water (w/o/w) double emulsion process that has been well-established in our lab, using camphor and ammonium carbamate as porogens.[6,21]

Two methods of PEGylation of the native 100% PLA MBs were used, as described previously.[6] Briefly, for the 5 wt% PEG-PLA MBs, an aliquot of 0.5g polymer was proportionally comprised of 5 wt% PEG-PLA (100 DL mPEG 5000 6CE, 67 mol% PLA, 33 mol% PEG, 69 kDa, Evonik Biomaterials, Essen, Germany) and 95 wt% PLA (100 DL 7E, 118 kDa, Evonik). Of the 0.5g total polymer mass, the proportion of PEG to PLA is 8.25mg (1.65%) PEG to 491.75mg (98.35%) PLA. For the 1 wt% LipidPEG MBs, 1,2-distearoyl-s*n*-glycero-3-phosphoethanolamine-*n*-[amino(polyethylene glycol)2000] (Avanti Polar Lipids, Alabaster, AL) was added to the initial phase of the w/o/w emulsion. The lipid was used as received and the chloroform was evaporated off under a stream of nitrogen gas before adding the appropriate weight of PLA polymer and methylene chloride to begin the double emulsion process.

2.2 MB Dox Loading

MBs loaded with Doxorubicin HCl (Dox, Tecoland, Irvine, CA) were generated by modifying the standard MB fabrication procedure to dissolve 15mg (3% w/w) of the chemotherapeutic agent in the polymer solution before the primary emulsion. This counterintuitive addition of hydrophilic drug to the organic phase was found to give considerably superior encapsulation results than addition to the aqueous phase, possibly due to the basic nature of the ammonium carbamate solution (data not reported). Drug was loaded into the standard (native, unmodified) MB and the MBs containing PEG-PLA during the emulsion process, prior to ligation of TRAIL. For LipidPEG MB, the TRAIL was ligated to the lipid prior to incorporation into the shell along with the Dox.

2.3 MB Functionalization with TRAIL

TRAIL (expressed in E. coli, MW 19.6kDa, Sigma Aldrich, St. Louis, MO) was ligated onto the native and 5 wt% PEG-PLA MB surface (and their Dox-loaded counterparts) via maleimide chemistry. The reaction uses an N-betamaleimidopropionic acid hydrazide (BMPH, Fisher Scientific, Pittsburgh, PA) spacer arm of 0.81nm in length, using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, Sigma) and n-hydrosulfosuccinimide (NHS, Sigma) to activate and catalyze the reactions.[22-25] For the 1 wt% LipidPEG MBs, TRAIL was ligated to the

LipidPEG molecule via maleimide chemistry prior to MB fabrication, and was incorporated into the polymeric shell during the emulsion process (along with Dox for the drug-loaded MBs).

2.4 MB Characterization

Cumulative dose and time response tests were performed in a custom-built acoustic testing system, using a 5MHz, 12.7mm diameter, single element US transducer (Panametrics, Waltham, MA) spherically focused at a length of 50.8mm, with a 6db bandwidth of 91%, and a pulse length of 1μs, as described previously.[4,6,13,21,26] These tests were performed in triplicate, and the results were reported as an average of these readings. MB diameter was measured before and after US insonation, by dynamic light scattering using a Malvern ZetaSizer Nano ZS Particle Size Analyzer, as described previously.[6] For post-insonation sizing, US-generated particles were collected after 15min of insonation at the conclusion of the acoustic time response testing. Readings were taken in triplicate and analyzed for average and standard error. Resonant frequency of the functionalized MBs was measured using a pulse-echo setup with a custom-built sample holder, equipped with an acoustically transparent window and an air-backed metallic reflector, and an exchangeable single element 12.7mm diameter unfocused transducer (Panametrics), as described previously.[6] Resonant frequency was determined as that at which attenuation reaches a minimum point on the frequency/attenuation curve.[27]

2.6 Statistical Analysis

All data are expressed as a mean value ± standard error of the mean calculated with Microsoft Excel (Microsoft Corporation, Redmond, WA). Statistically significant differences for multiple groups were determined using a one-way ANOVA with Bonferroni correction for multiple comparisons and Tukey's multiple comparison post-test when appropriate. Differences were evaluated across all groups, and also within each group for more robust analysis. Statistical significance between individual groups for pre- and post-US size comparison was determined using a matched pairs Student's t-test. All statistical testing was done using Prism 7 (GraphPad,

San Diego, CA) using α=0.05 significance level. Error bars were displayed as standard error about the mean (SEAM).

3. Results

3.1. Physical Characterization: MB Size Analysis Pre- and Post-Insonation

The strength of acoustic signal reflected from MBs is largely influenced by the scattering cross-section and shell elasticity, both functions of the agent composition and diameter.[28-31] The scattering cross section is a function of the differences between the material properties of the scatterer and the surrounding medium.[31] All MB formulations had a pre-insonation average diameter between 1-3μm (Figure 1), which is well within the acceptable range (<6μm) for clear passage through the vasculature, resonance in the clinical frequency range, and susceptibility to radiation forces.[32,33] US-triggered size reduction was significant ($p<0.0001$) for each tested agent, and the n-Sh produced by insonation-induced shattering all had an average size within the range demonstrated for active extravasation in tumors (400-700nm, shown in Figure 1 as the area between the dotted lines).

Dox and TRAIL loading of the 100 wt% PLA group (Figure 1A) resulted in decreased average diameter compared to native control MBs ($p<0.0001$), ranging from 1.43±0.04 to 1.71±0.06μm compared to native 100% PLA MBs (2.41±0.10μm), possibly due to increased amounts of debris particularly in the 100% PLA MB groups caused by the additional processing. However, particle size distribution, measured by PDI was not significantly different for any of the 100% PLA groups, ranging from 0.184 to 0.214, possibly due to the lack of sensitivity of DLS to the nano range. In the 5 wt% PEG-PLA group (Figure 1B), average MB diameter increased from native, unmodified 5 wt% PEG-PLA (1.78±0.06μm) to 5 wt% PEG-PLA Dox MB (2.24±0.07μm) ($p<0.0001$). This change is likely also due to interaction between polymer groups while accommodating Dox loading, altering the shell properties. MB diameter further increased with TRAIL ligation (2.35±0.06μm, $p<0.0001$), likely due to the size of the TRAIL molecule coupled with shell effects caused by the maleimide ligation in an aqueous solution. Our lab previously

showed an increase in MB size immediately upon introduction to an aqueous environment, indicating MB swelling.[15] All 5 wt% PEG-PLA MBs had a wider size distribution than 100% PLA MBs, with PDI ranging from 0.258 to 0.346. Similarly, 1 wt% LipidPEG Dox MBs (1.45±0.06μm) were larger than native 1 wt% LipidPEG MBs (1.24±0.04μm, p=0.0009) (Figure 1C) attributed to rearrangement of polymer chains and lipid tail groups to accommodate Dox encapsulation. Average particle size decreased with TRAIL ligation, both for 1 wt% LipidPEG TRAIL MB (0.89±0.02μm, p<0.0001) and 1 wt% LipidPEG Dox TRAIL MB (1.04±0.03μm, p=0.0029) compared to their unmodified native counterpart. Steric hindrance introduced by the TRAIL molecules preventing PEG folding into the mushroom formation could account for this increase.[34-36] Similar to the 5 wt% PEG-PLA group, all 1 wt% LipidPEG MBs exhibited an increased PDI (0.262-0.352), indicating that these agents have a broader size distribution. Since insonation resulted in production of particles within the 400-700nm range, it is important to note that shell modification with Dox, TRAIL, and a combination of both does not prevent shattering into n-Sh for US-driven drug delivery. The US-produced drug- and ligand-loaded n-Sh would be of small enough size to pass through the pores in the leaky tumor vasculature (400-780nm) and reach the targeted tissue for effective therapy.

3.2 Acoustic Properties

The preservation of adequate acoustic properties upon modification of the MBs is essential, not only because the agent greatly increases the contrast of US images (in our case better delineation of tumor tissue) upon passage through the vasculature, but also to retaining the cavitation-induced generation of drug-loaded n-Sh. We studied three shell types (PLA, PEG-PLA, and LipidPEG), and three manipulations (Dox encapsulation, TRAIL ligation, and Dox+TRAIL). A complete summary of the effects of the various shell modifications is given in Table 1. As clearly seen in the table, both shell properties and the manipulations had an effect on acoustic properties which will be discussed in the relevant sections to follow.

3.2.1 Acoustic Enhancement

For comparison and evaluation of feasibility for future *in vivo* studies, we have shown that acoustic backscatter measured *in vitro* down to 15dB can give a detectable contrast-enhanced image *in vivo.*[12] Figure 2 shows the results of *in vitro* monitoring of the acoustic backscatter at 37°C as a function of MB dose as a function of shell composition. These results are plotted compared to the unmodified, native shell. While the general shape of the dose response curve was similar in all cases (rising to a maximum), two salient features varied with treatment: the dose required to reach the maximum backscatter and the value of that maximum (dB). The required dose (Table 1, column 2; and shown in Figure 2) nearly doubled in response to all modifications, rising from 7.5μg/mL to 13.5μg/mL or higher. The maximum achievable enhancement (Table 1, column 3) varied considerably with treatment, being most affected by inclusion of TRAIL, dropping to a low of 10.53±0.85 dB when TRAIL was added to the 100% PLA. However, a maximum enhancement of 14.25±0.82 dB was achieved when TRAIL was incorporated into the LipidPEG formulations. It should be noted that TRAIL is pre-attached to the lipid prior of MB formation, avoiding the need for a second exposure to an aqueous environment during the surface ligation procedure. In the case of the 1 wt% LipidPEG TRAIL MB, the maximum echogenicity rose to 16.13±0.80 dB upon addition of Dox. In fact, increases in echogenicity when Dox was encapsulated within the shell was a trend across almost all cases.

There were no significant changes to maximum enhancement for the 1 wt% LipidPEG MB when functionalized (Figure 2C), suggesting that this platform is the most versatile for modifications. The relative integrity of these agents in response to manipulation is attributed to the introduction of the lipid molecule in the w/o/w emulsion process, contributing to the elasticity of the polymeric shell and avoiding subsequent modification through re-introduction to aqueous solutions.

Table 1 shows that there were no significant differences ($p>0.4050$) in maximum enhancement between any of the three unmodified native MBs (100% PLA, 5 wt% PEG-PLA, and 1 wt% LipidPEG), establishing a good baseline for comparison. When MBs were modified to encapsulate Dox within the polymer shell, 5 wt% PEG-PLA Dox MBs clearly exhibited the highest acoustic enhancement (19.91±0.51 dB), and was significantly higher than both 100% PLA Dox MB (16.23±0.59 dB, $p=0.0292$) and 1 wt% LipidPEG Dox MBs (16.03±1.01 dB, $p=0.0158$). TRAIL ligation negatively affected the enhancement, and resulted in 1 wt% LipidPEG TRAIL MBs having the highest maximum cumulative enhancement (14.25±0.82 dB), which was significantly higher than 100% PLA TRAIL MBs (10.53±0.85 dB, $p=0.0259$). The observed trends in acoustic enhancement likely also reflect the fact that TRAIL is pre-ligated to the LipidPEG molecule before the double emulsion process, while in the 100% PLA and 5 wt% PEG-PLA agents pre-formed capsules are reintroduced to an aqueous environment for TRAIL ligation.

3.2.2 Acoustic Stability

To investigate the instability of the MBs within an US beam, a prerequisite for *in situ* n-Sh formation, *in vitro* time response curves were constructed in the acoustic setup, using two acoustic pressures, one at a lower mechanical index (MI) useful for imaging (0.152 at a peak negative pressure (PNP) of 0.4 MPa), and the second at a higher MI useful for inducing inertial cavitation (0.193 at a PNP of 0.94 MPa). As with the dose response data, all plots had a similar shape (Figure 3). At the higher pressure, as expected, all agents had a short half-life ($t_{1/2}$) of between 1-2 minutes (Table 1, column 4). At the lower pressure, the LipidPEG bubbles were the least stable in the US beam recording half-lives of approximately 8 minutes (Table 1, column 3). At this pressure, addition of LipidPEG to the shell had the same effect on the acoustic $t_{1/2}$ as did addition of Dox to the native PLA agent. The 1 wt% LipidPEG Dox TRAIL MB were less stable than 100% PLA Dox TRAIL MB ($p=0.0008$), suggesting that these shells are more easily

disrupted possibly due to the shear stresses of the long molecules extending from the MB surface and shell instability at the point of lipid tail incorporation.

Introduction of Dox into the native 100% PLA MB caused an almost 50% drop in stability, suggesting that Dox encapsulation disrupts the shell structure making it more flexible to still allow for oscillations but with less stability;[37,38] stability within the US beam was restored for both TRAIL and Dox TRAIL manipulations. A similar pattern was seen for the PEG-PLA series. The reported normalized acoustic half-life of the TRAIL and Dox TRAIL MB is independent of the initial magnitude of the enhancement. During ligation with TRAIL, the 100% PLA and 5 wt% PEG-PLA unloaded and Dox-loaded MB are under aqueous conditions, and will experiences some hydrolysis and structural changes, which alters the shells making them less susceptible to cavitation (and therefore, more stable within the US beam).[37-39] Since the 1 wt% LipidPEG MB do not undergo this process for TRAIL ligation, their stability is relatively unchanged regardless of manipulation.

3.2.3 Resonant Frequency

MB and microbubbles at resonance are most likely to undergo inertial cavitation, leading to collapse and shattering. The resonant frequency of each functionalized MB was determined experimentally, in a manner similar to Forsberg et al.,[27] taking the minimum point from the graph plotting attenuation vs. frequency curves (Figure 4).

Loading Dox into the parent (native) 100% PLA shell caused a slight downwards shift in resonance to 4.04MHz, from 4.56MHz. Both TRAIL and PEG-PLA incorporation into the parent PLA shell cause increases in resonant frequency from 4.56MHz to 7.24MHz and 7.84MHz, respectively (Figure 4A and B), and this resonant frequency remained high when Dox was also added (Table 1, column 5). However, in the case of LipidPEG MBs, in which both the PEG component and TRAIL-ligated PEG are “hooked” into the shell in the initial w/o/w emulsion, resonant frequency remains more or less constant, and in line with the parent 100% PLA MBs.

4. Discussion

In almost all cases, an increase in echogenicity was observed when Dox was encapsulated within the polymeric shell. This is likely due to hydrophilic interactions between the polymer blocks and the hydrophilic Dox molecules, as investigated by Nahire, affecting the rigidity and cavitation of the resulting Dox-loaded MB.[28,40] Studies also suggest that Dox encapsulation disrupts the shell structure making it more flexible to still allow for oscillation.[38] In terms of the variable echogenic response in the TRAIL-ligated MBs, the additional exposure to the aqueous environment may cause swelling and hydrolysis of the PLA-based shells, leading to structural changes and minute amounts of material degradation that influence the MB echogenicity.[39] When considering tuning the MBs for personalized treatment, the 1 wt% LipidPEG shell type emerges as the better candidate for ligand attachment, while the 5 wt% PEG-PLA shell type is better suited for drug encapsulation; both shell types have superior immunogenic reduction to the 100% PLA shell type.[6] These results demonstrate that PEGylated MBs can be functionalized to carry cancer therapeutics while retaining acoustic responsiveness, presenting the advantage of reduced immune response in combination with targeted treatment compared to the native, unmodified MBs. This study particularly evaluated Dox due to its synergistic relationship with TRAIL, but our versatile polymeric agents can be modified to encapsulate a hydrophilic or hydrophobic drug, or a combination of drugs, to treat a variety of tumor types.[9] While echogenic response is diminished by functionalization in most cases, the resulting functionalized MB are still capable of interacting with US under conditions similar to those used in a clinical setting.[12] Results suggest that 1 wt% LipidPEG shell type is the most versatile for adaptation via TRAIL-ligation (native or Dox-loaded), while 5 wt% PEG-PLA best retains acoustic behavior for Dox loading without addition of TRAIL.

Similarly, shifts in resonant frequency indicate changes to the shell elasticity and stiffness, affecting the ability of the MBs to cavitate and resonate within the US beam.[29,31,37,38] The TRAIL-ligated MBs also had reduced average diameters, compared to the non-ligated MBs, further

affecting the resulting resonant frequency as diameter is inversely related to resonance.[28-31,37,38,41] Such a shift in resonant frequency explains the reduced echogenicity observed in our acoustic evaluations, as the transducer bandwidth may not effectively insonate the MBs resulting in reduced oscillations and cavitation. Since the resonant frequency of the1 wt% LipidPEG group showed minimal dependence on shell modification, this shell material appears to be the most versatile for modification to desired applications, especially drug-loaded MB decorated with the TRAIL targeting ligand.

5. Conclusions

We believe that this study is the first to investigate the ramifications of combined drug (Dox) encapsulation and targeting ligand (TRAIL) ligation, both separately and in combination, on the acoustic behavior of polymeric MBs with three different shell types. The results demonstrate the combined influence of shell materials, particularly block co-polymers and lipid tails, and modifications such as drug incorporation and ligand attachment. The insights allow for careful tuning of the properties to adapt for the many different scenarios encountered in US-driven drug delivery to diverse classes of cancerous tumors.

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Figure 1: Average particle size of MBs pre-insonation and post-insonation (MI 0.193 at 0.94MPa PPP). A) Measurements of the 100% PLA group, ***p<0.0001, B) Measurements of the 5 wt% PEG-PLA group, ***p<0.0001, C) Measurements of the 1 wt% LipidPEG group, **p<0.01, ***p<0.0001. Dotted lines represent 400-700nm range, the desired range for extravasation of US-produced n-Sh. Error bars represent SEAM, n=5.

Figure 2: Effect of treatment on cumulative dose response curves within a given shell type (n=5, error bars=SEAM, ***p<0.0001). A) 100% PLA MB group, B) 5 wt% PEG-PLA MB group, C) 1 wt% LipidPEG MB group.

Figure 3: Acoustic results plotted as normalized time response curves (n=5, error bars=SEAM). Dotted line represents acoustic half-life. A) 100% PLA group, *p=0.0439 for 100% PLA MB to 100% PLA TRAIL MB, *p=0.0497 for 100% PLA Dox MBs to 100% PLA TRAIL MBs, **p=0.0014, ***p<0.0001, B) 5 wt% PEG-PLA group, C) 1 wt% LipidPEG group.

Figure 4: Effect of shell composition on attenuation (dB/cm) vs. frequency (MHz). Solid line represents measurements taken with 5MHz unfocused transducer, and dotted line represents measurements taken with 10MHz unfocused transducer. PRF=100Hz, Damping Level=3, Gain=0. A) Measurements of the 100% PLA group, showing a clear shift in resonant frequency, B) Measurements of the 5 wt% PEG-PLA group, which were affected by increased debris and changes in MB morphology, C) Measurements of the 1 wt% LipidPEG group, showing a relatively unchanged resonant frequency across modification types.