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Microbubble–sonosensitiser conjugates as therapeutics in sonodynamic therapy†

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A Rose Bengal sonosensitiser has been covalently attached to a lipid microbubble and the resulting conjugate shown to produce higher levels of singlet oxygen, enhanced cytotoxicity in a cancer cell line and a greater reduction in tumour growth than the sonosensitiser alone.

Microbubbles (MBs) are small (typically 1–8 μm in diameter) gas filled microspheres that are currently used as contrast agents in ultrasound based imaging. Clinical ultrasound uses MBs to help opacify blood filled cavities and to visualise tissue perfusion. The core of a MB is filled with an inert gas with low aqueous solubility such as perfluorocarbons, coated with a stabilising shell consisting of different materials such as phospholipids, proteins (especially albumin) and biocompatible polymers.[1] The behaviour of MBs depends on the amplitude of ultrasound to which they are exposed. At low acoustic pressures the MB oscillates in a relatively symmetrical linear fashion.[2] At high acoustic pressures the MB undergoes forced expansion and compression which results in destruction of the MB by either outward diffusion of the gas during the compression phase, defects in the stabilising shell, or by a complete fragmentation of the gas and shell.[3] The ability to selectively destroy MBs using an ultrasound stimulus of appropriate energy has led to them being studied as potential drug/gene delivery vehicles in recent years.[3–5]

Sonodynamic therapy (SDT) involves the use of ultrasound, a sonosensitising drug and molecular oxygen to generate reactive oxygen species (ROS). While the mechanism for ROS production in photodynamic therapy (PDT) is well understood, the mechanism for the generation of ROS in SDT is less clear. One suggestion is that the process of ultrasound inertial cavitation, which involves the formation, oscillation and collapse of gas filled bubbles in samples irradiated with ultrasound is responsible for initiating the generation of ROS in SDT.[6] When this cavitation phenomenon becomes dominated by inertial forces, the bubbles collapse violently leading to sonoluminescence emission.[7] This luminescence emission may subsequently excite the nearby sonosensitiser by an energy transfer process resulting in the generation of ROS by the very same mechanism as in PDT. Another possible explanation is that sonosensitiser drugs in the vicinity of collapsing bubbles experience such high local temperatures that ROS are generated through pyrolysis reactions.[8]

The potential benefits of attaching sonosensitising drugs to microbubbles are threefold: (1) it enables the site specific delivery of the sonosensitisers by selectively destroying them with a non-invasive ultrasonic stimulus. (2) Due to the tissue attenuation of ultrasound, deep seated tumours can be accessed. This is a particular advantage over PDT where the low tissue penetration capability of visible light limits this technique to the treatment of superficial tumours.[9] (3) The close proximity of the sonosensitiser to the MB should enhance the possibility of ROS generation by either of the aforementioned mechanisms.[7,8] Combined, these benefits mean that a completely non-invasive approach for the treatment of deep seated tumours is possible.

In this manuscript we covalently attach a Rose Bengal sonosensitiser to the surface of a lipid based MB using a carbodiimide based coupling protocol. We evaluate the ability of the resulting conjugate to produce singlet oxygen and determine its cytotoxic potential in a cancerous cell line upon ultrasound irradiation. Finally, we examine the cytotoxic effect of this conjugate *in vivo* using a human prostate tumour model in SCID mice by measuring the % tumour growth before and after ultrasound irradiation.

Lipid MBs were prepared by sonication of an aqueous dispersion of the lipid-based reagents in the presence of a perfluorobutane gas stream (see ESI†). Amine functionality in the MB shell was accomplished by the incorporation of distearoylphosphatidyl ethanolamine-polyethylene glycol-amino (DSPE-PEG) at 5% of the total lipid concentration (Scheme 1). These amino functionalised MBs were characterised by optical microscopy and observed to have an average diameter of 1.7 ± 0.5 μm. To enable covalent amide bond attachment of the sonosensitiser to the MB shell it was first necessary to derivatise the commercially available Rose Bengal sodium salt (**RBNa**) with carboxylic acid functionality. This was accomplished by the nucleophilic substitution reaction between 8-bromooctanoic acid and **RBNa** (Scheme 2).[10] This product (**RB1**) was then covalently attached to the pendant amino groups of the MBs using standard carbodiimide coupling techniques (Scheme 2).[11] After purification by centrifugation the **MB–RB** conjugates were isolated as a pink coloured milky suspension that floated on top of the PBS solution.

Optical microscopy analysis showed the presence of pink coloured bubbles with a concentration of 1×10^9 MBs per ml

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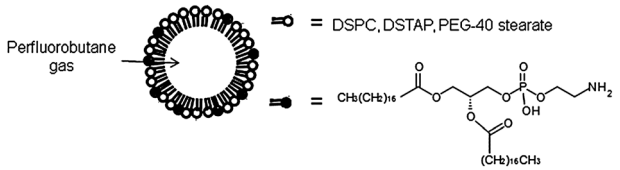
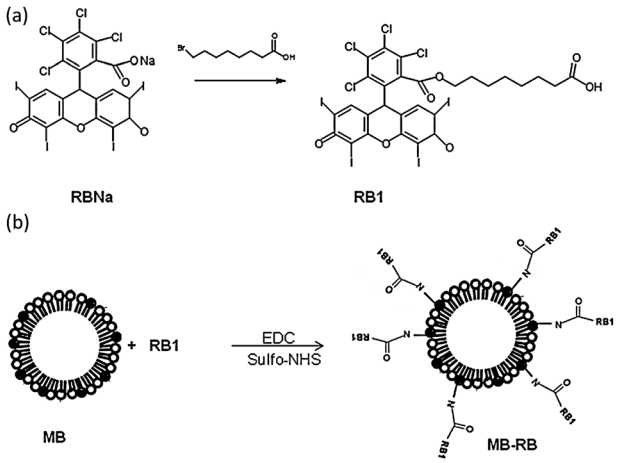


Scheme 1 Schematic representation of the lipid microbubbles used in this study. DSPC = distearoylphosphatidyl choline; PEG-40 stearate = (polyethylene glycol)-40-stearate. Also shown is the chemical structure of DSPE-PEG (distearoylphosphatidyl ethanolamine-polyethylene glycol)-amino.



Scheme 2 Synthesis of (a) **RB1** and (b) the **MB–RB** conjugate.

(Fig. S1, ESI†). Using a standard calibration curve, we determined the concentration of the attached Rose Bengal to be 92.7 μM. This represents a grafting efficiency of **RB1** to the MB of 37.5%. In addition to the **MB–RB** conjugate a control reaction was also performed where a solution of **RB1** was mixed with the MBs under the very same conditions and subjected to the same purification protocol, without the presence of the coupling agents EDC and sulfo-NHS. The purpose of this control reaction was to determine if there would be any non-covalent interactions between **RB1** and the MBs through a self assembly process.

To determine the potential of the **MB–RB** conjugate to produce singlet oxygen under ultrasound stimulation we utilized the photo-oxidation of 1,3-diphenylisobenzofuran (DPBF) as a quantitative method.[12] Specifically, 2 mL of the **MB–RB** conjugate was added to an aerated solution of DPBF (10 μM) in an EtOH : H_2O (50 : 50) solvent system. The solution was then irradiated with ultrasound delivered by a Sonidel SP100 sonoporator (emitting at a frequency of 1 MHz, using an power density of 1.5 W cm^{-2} for 60 min and a 50% duty cycle at a pulse repetition frequency of 100 Hz). This procedure was repeated for the control MB sample and for un-conjugated **RB1** alone at a similar concentration to that present in the **MB–RB** conjugate. The results are shown in Fig. 1a and reveal a significant loss in the DPBF absorbance at 410 nm for the **MB–RB** conjugate indicating significant singlet oxygen production. However, no noticeable reduction in DPBF absorbance was observed for the control MB sample suggesting the presence of covalent bonds between the MB and sonosensitiser are necessary for high yields of singlet oxygen to be produced. Surprisingly though, the solution containing only **RB1** (*i.e.* the sonosensitiser alone at the same concentration as that present in the **MB–RB** conjugate) also failed to produce significant quantities of singlet oxygen upon ultrasound irradiation. Initially, we assumed this experiment would function as a positive control for the singlet oxygen potential of **RB1** upon ultrasonic activation. Indeed, the sodium salt of Rose Bengal (**RB**) did produce comparable singlet oxygen levels as the **MB–RB** conjugate when present at a much higher concentration[13] (5 μM). This suggests that at a concentration of 1.23 μM (which was the concentration of **RB1** in the **MB–RB** conjugate), the yield of singlet oxygen produced by **RB1** alone is too low to be measured by this assay. However, it does indicate a powerful synergistic relationship between the **MB** and **RB1** in **MB–RB** that significantly enhances the singlet oxygen capability of the sonosensitiser. We postulate that this enhanced singlet oxygen production results from the ultrasound mediated inertial cavitation of the MBs that ultimately results in their collapse. The collapsing bubble facilitates the production of singlet oxygen by either a sonoluminescence or pyrolysis (or both) mediated process mentioned earlier. Indeed, Gaitan *et al.*, have previously demonstrated that bubbles trapped in a standing ultrasonic wave produced repeated flashes of sonoluminescence attributed to the occurrence of inertially dominated bubble collapse.[14,15] Shi *et al.* experienced a similar phenomenon when using a very dilute suspension of a commercially available microbubble contrast agent.[16] As the efficiency of an energy transfer process is distance dependent,[17] the covalent attachment present in **MB–RB** ensures a close MB–sonosensitiser distance enabling efficient sonosensitiser excitation upon ultrasound activation of the MB–sonosensitiser conjugate. Similarly, pyrolysis mediated production of singlet oxygen would also benefit from a close MB–sonosensitiser distance, as one would expect the local temperature to be greater closer to the surface of the collapsing MB. We are currently developing methods to ascertain which of these two mechanisms most likely contribute to singlet oxygen production in these systems.

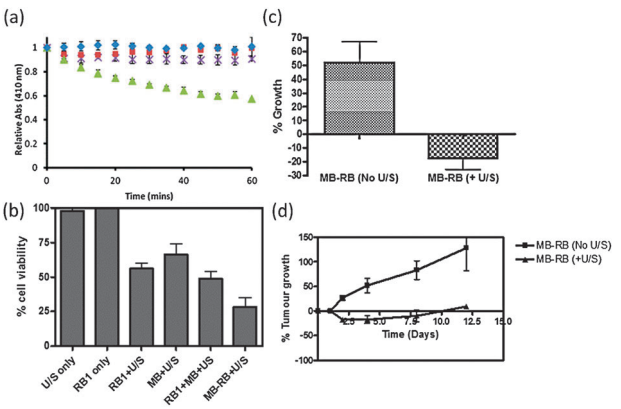


Fig. 1 (a) Plot of relative absorbance of DPBF at 410 nm against time for: **MB–RB** conjugate (green △), control MBs (i.e. no EDC/S-NHS used) (red □), MBs alone (purple X) and **RB1** alone (blue ◊). (b) Plot of % cell viability for RIF-1 cells: exposed to US only (**U/S** only); incubated with **RB1** without US (**RB1** only); incubated with **RB1** and US (**RB1** + **U/S**); with MBs and US (**MB** + **U/S**); incubated with **RB1** and MB non-covalently linked and US (**RB1** + **MB** + **U/S**); and incubated with the covalently linked **MB–RB** conjugate exposed to US. (c) Plot of % tumour growth against time for mice treated with the **MB–RB** conjugate with (△) and without (□) US treatment. (d) Bar chart representing the % change in tumour growth for treated and untreated tumour 4 days post-treatment.

To determine if the singlet oxygen generated would have the desired cytotoxic effect in tumour cells, we carried out a similar experiment using RIF-1 cells as a target in a tissue culture-based bioassay. RIF-1 cells were cultured in 96-well plates and **MB–RB** was added to selected wells at a MB concentration of 2×10^7 ml^{-1}. These were then treated with ultrasound for 30 s, using a frequency of 1 MHz, an ultrasound power density of 1.5 W cm^{-2} and a duty cycle of 50% (pulse frequency = 100 Hz). Control wells containing **RB1** alone and those containing MBs and **RB1** at the same concentration as in the **MB–RB** conjugate were used for comparative purposes. We also included controls for the effect of ultrasound alone and MBs alone for completeness. Following irradiation the cells were incubated for 24 hours before the viability was determined using an MTT assay.[18] The results show a 72% reduction in cell viability for the **MB–RB** conjugate upon ultrasound irradiation while the un-conjugated MB/**RB1** solution was significantly less effective with a 51% reduction in cell viability. Given **RB1** alone produced a similar cytotoxic effect at 44% reduction, this further emphasises the importance of a covalent interaction between the MB and sonosensitiser for enhanced cytotoxicity. The fact that **RB1** alone produced a cytotoxic effect when present at the same concentration as in **MB–RB** while it produced negligible singlet oxygen in the DPBF assay may be due to two reasons (i) the DPBF assay is not sufficiently sensitive to measure levels of singlet oxygen that are cytotoxic in a cellular environment or (ii) other ROS in addition to singlet oxygen may be generated that are not detected by the DPBF assay. Nonetheless, these results validate those from the DPBF assay in that a significant enhancement in toxicity is obtained by covalent attachment of the sononsensitiser to the MB.

To determine the therapeutic efficiency of the **MB–RB** conjugate *in vivo*, tumours were induced in BALB/c SCID mice using the modified human prostate cell line LNCaP-Luc. Once the tumours were 1.24 cm^3, a 30 μl aliquot of the **MB–RB** conjugate (2×10^8 MB ml^{-1}) was injected into the tumour. In these experiments, intratumoral injection was chosen as the administration route in order to preclude variables resulting from systemic delivery. The tumours were then treated with ultrasound for 3 min using a frequency of 1 MHz, an ultrasound power density of 3.5 W cm^{-2} and 30% duty cycle (100 Hz pulse frequency). Control mice that were administered the **MB–RB** conjugate but not exposed to ultrasound irradiation were also used for comparative purposes. The results are shown in Fig. 1c and d and reveal a significant reduction in tumour size for those animals treated with the **MB–RB** conjugate and ultrasound compared to the **MB–RB** conjugate alone. In fact, 4 days after treatment, tumours on animals treated with the **MB–RB** conjugate and ultrasound actually regressed and were found to be 18% smaller than the original pre-treatment size (Fig. 1d). On the other hand tumours on animals treated with **MB–RB** in the absence of the ultrasound stimulus had increased in size by 50% on day 4. It was interesting to note that it was not until day 10 that tumours treated with **MB–RB** and ultrasound reached their pre-treatment size, whereas those treated with **MB–RB** in the absence of ultrasound had increased to 100% that of the pre-treatment tumour size (Fig. 1c). Essentially these results dramatically demonstrate the therapeutic potential of our approach and highlights the necessity for a combination of ultrasound and the conjugate at the target site. We demonstrate here that the conjugate has no effect on tumour growth in the absence of the ultrasound stimulus and it should also be noted that from previous studies, the ultrasound conditions employed had no effect on tumour growth.[19,20] Since neither the conjugate nor the stimulus exhibit toxicity, the system essentially comprises the best attributes of a targeted therapeutic system.

In summary, a MB–sonosensitiser conjugate has been prepared and observed to produce significant quantities of singlet oxygen and be more cytotoxic to a cancerous cell line when irradiated with ultrasound compared to the un-conjugated sonosensitiser at the same concentration. In addition, ultrasound irradiation of animals treated with the **MB–RB** conjugate significantly reduced tumour growth when compared to those that received the drug but no ultrasound. We attribute these effects to either a sonoluminescence or pyrolysis mediated production of ROS. Either of these processes would benefit from a close MB–sonosensitiser separation as offered by the covalent linkage present in **MB–RB**. This approach offers the potential to deliver sonosensitiser drugs to deep seated tumours and activate them in a non-invasive manner. Furthermore, the covalent attachment of the sonosensitiser not only improves the singlet oxygen quantum yield but also reduces the possibility of the sonosensitiser leaching from the bubble prior to being activated at the target site. This not only improves the likelihood of a greater proportion of the drug reaching its target site, but also reduces the inadvertent activation of the sonosensitiser with ambient light,[21] as Rose Bengal is also known to be a potent photosensitiser.

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