Electronic Supplementary Material (ESI) for Chemical Communications

Microbubble – Sonosensitiser Conjugates as Therapeutics in Sonodynamic Therapy

SUPPORTING INFORMATION

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Materials and Methods.

Reagents: Rose Bengal sodium salt, EDC, NHS, diphenylisobenzofuran, MTT, PEG40-stearate and ethanol were purchased from Aldrich at the highest grade possible. Distearoyl phosphatidylcholine, 1,2-distearoyl-3-trimethylammoniumpropaneweere purchased from Avanti USA. NMR spectra were recorded on a Varian 500 MHz spectrometer. ESI-MS characterisation of **RB1** was achieved using a LCQTM quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, California, USA) utilising electrospray ionisation (ESI). Absorbance measurements were recorded on a Varian Cary 50 Spectrometer using 10 mm quartz cuvettes.

Preparation of amino-functionalised MBs: MBs were prepared by sonication of an aqueous dispersion of the lipid-based reagents in the presence of a perfluorobutane gas stream as described by Nomikou *et al.*[1] The MBs were stabilised by the inclusion of a polyethylene

glycol-lipid conjugate in the shells. The molar ratio of each lipid-based reagent in the MB shell was 51% DSPC (distearoylphosphatidyl choline), 44% PEG (polyethylene glycol)-40-stearate and 5% DSPE-PEG (disteroylphosphadityl ethanolamine-polyethylene glycol)-amino. The preparation was adjusted to a concentration of 1 x 10^9 MBs/ml using PBS (phosphate buffered-saline).

***Preparation of carboxylic acid Rose Bengal (1)[2]*:** Rose Bengal sodium salt (1.0g, 9.83 x 10^{-4} mol), 8-bromooctanoic acid (0.66g, 2.95 x 10^{-3} mol) and anhydrous DMF (10 mL) were refluxed at 80°C for 7 hrs. The solvent was evaporated under reduced pressure and the resulting residue stirred in diethyl ether (100ml) for 18h at 25°C. The solution was filtered and the solid residue isolated. The residue was then stirred in water (100ml) for 18 h at 25°C, the solution filtered and the product isolated as a red powder. (Yield = 0.68g, 62.1%). ^{1}H NMR (500 MHz, DMSO-d_6): *0.80 (m, CH_2, 2H), 1.00 (m, CH_2, 2H), 1.10(m, CH_2, 4H), 1.40 (m, CH_2, 2H), 2.20 (t, CH_2COOH, 2H), 3.90 (t, OCH_2,2H), 7.50(s,ArH,2H), 11.90 (s, COOH, 1H).* ^{13}C NMR (125 MHz, DMSO-d_6): δ 25.0, 25.5, 28.2, 28.8, 28.9, 34.2, 66.6, 76.4, 97.8, 110.8, 129.2, 130.4, 132.3, 134.8, 135.4, 136.6, 139.8, 157.5, 163.9, 172.2, 174.9; +ESMS: expected 1114.6 Da, found 1115.8.

***Conjugation of RB1 to amino functionalised MB's**:* A saturated stock solution of **1** was prepared by dissolving 10 mg (0.045 mmol) of **1** in PBS (5mL) and sonicating for 30 min. Any un-dissolved **1** was removed by passing the solution through a 100 nm sterile filter and the concentration of the stock solution determined as 0.6 mM using a standard calibration curve. Similarly a stock solution of N-hydroxysulfosuccinimide sodium salt (S-NHS) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) were prepared by dissolving S-NHS (10 mg, 9.2 mM) and EDC (9 mg, 9.4 mM) in 5 ml of PBS. The resulting solution was passed through a 100 nm sterile filter. **1** (0.82 ml, 0.49 µmol) was then placed in a 15 ml centrifuge tube and 0.15 ml of the S-NHS / EDC solution in PBS (~0.71 µmol of

both S-NHS / EDC) was added. The solution was vortexed for 5 minutes, the MB suspension (1 ml, 0.24 µmol amine groups) added and the mixture stirred for 30 minutes. The resulting suspension was centrifuged at 700 rpm for 5 min and the **MB-RB** conjugate removed as a pink coloured milky suspension floating on top of the liquid layer. The **MB-RB** conjugate was washed twice with PBS (4 ml), centrifuged and isolated by decanting the top layer. The **MB-RB** conjugate was then used directly in the *in vitro* and *in vivo* experiments. The final concentration of **RB1** in the **MB-RB** solution was determined as 92.7 µM using standard calibration.

Determination of singlet oxygen generation of MB-RB conjugate: An EtOH:H_2O (50:50 v/v) (150 mL) solution was prepared containing **MB-RB** ([MB] = 2 x 10^9, [RB1] = 1.23 µM) and DPBF (10 µM). The solution was aerated for 10 mins before being irradiated with ultrasound for 60 mins using a Sonidel sonic probe (2.6 W/cm^2, 50 % duty cycle and pulse repetition rate of 100 Hz). Aliquots were removed and their absorbance recorded every 5 minutes at 410 nm using a Cary Absorbance spectrometer.

Cytotoxicity evaluation of MB-RB conjugates*:* A mouse radiation-induced fibrosarcoma cell line (RIF-1) was used as a target cell line[3]. The cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum at 37°C in a humidified 5% (v/v) CO_2 atmosphere. These cells were plated into the wells of a 96-well tissue culture plate at a concentration of 2 x 10^4 cells per well and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. The media was then removed from each well and replaced with 50 µl of the conjugate **MB-RB** preparation at a concentration of 2 x 10^7 MB / ml. These were then treated with ultrasound (**MB-RB** + U/S) 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 preparations consisted of (i) cells treated with ultrasound in PBS (U/S alone); (ii) cells treated with **RB1** at a concentration equivalent to that bound to the MBs in **MB-RB** (RB1 only); (iii)

as in (ii) with ultrasound (RB1 + US) (iv) MBs at a concentration equivalent to that used in **MB-RB** with ultrasound (MB + US) (v) cells treated with ultrasound in the presence of an un-conjugated MB and **RB1** mixture (RB1 + MB + U/S). Following treatment, samples were incubated for 30 min at 37°C in a humidified 5% CO_2 atmosphere. Wells were then emptied, washed with PBS and 200 µl aliquots of serum-containing medium were dispensed into each well. Following an overnight incubation at 37°C, cell viability was determined using an MTT assay[4].

Cytotoxicity evaluation in vivo: Tumours were generated using a modified form of the LNCaP human prostate cell line LNCaP-Luc[5] and this was cultured in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum, 100mM HEPES and 5 mM glucose at 37°C in a humidified 5% (v/v) CO_2 atmosphere. Every second change of medium contained geneticin at a concentration of 300 µg/ml to maintain selective pressure. In order to induce tumour formation, 5 x 10^6 cells in 100 µl aliquots of Matrigel® were injected subcutaneously on the dorsum of BALB/c SCID mice (8 weeks old). In all experiments animals were treated humanely and in accordance with licensed procedures under the UK Animals (Scientific Procedures) Act, 1986. When the tumours had reached an average size of 1.24 cm^3, a 30 µl aliquot of the **MB-RB** conjugate (2 x 10^8 MB/ml) was injected into each tumour. Animals were subsequently 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 animals were injected with the conjugate but did not receive ultrasound. At various time intervals post treatment, tumour volumes were calculated using volume = $4/3(\pi r^3)$ where r was the tumour radius determined from 3-leg measurements taken at the indicated times.

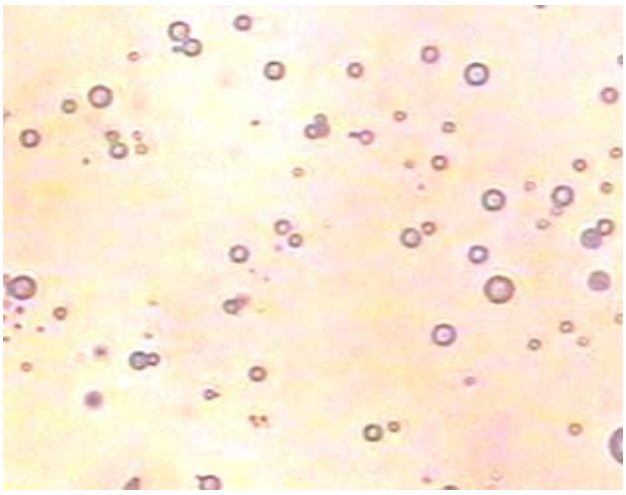

Figure S1 Examination of **MB-RB** conjugates, using a Nikon Eclipse E400 fluorescence microscope (Japan), fitted with a x40 objective lens. Photomicrographs were acquired using a digital camera interfaced with a PC operating the Andor™ iQ Standard Video-Meteor II software system (v.1.3) (Andor™ Technology Plc., UK).

References

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