Journal of Controlled Release 262 (2017) 192–200

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

# Magnetically responsive microbubbles as delivery vehicles for targeted sonodynamic and antimetabolite therapy of pancreatic cancer

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ARTICLE INFO

*Keywords:*
Microbubbles
Magnetic targeting
Drug delivery
Hypoxia
5-Fluoruracil
Rose Bengal
Sonodynamic therapy
Antimetabolite therapy
Pancreatic cancer

ABSTRACT

Magnetically responsive microbubbles (MagMBs), consisting of an oxygen gas core and a phospholipid coating functionalised with Rose Bengal (RB) and/or 5-fluorouracil (5-FU), were assessed as a delivery vehicle for the targeted treatment of pancreatic cancer using combined antimetabolite and sonodynamic therapy (SDT). MagMBs delivering the combined 5-FU/SDT treatment produced a reduction in cell viability of over 50% when tested against a panel of four pancreatic cancer cell lines in vitro. Intravenous administration of the MagMBs to mice bearing orthotopic human xenograft BxPC-3 tumours yielded a 48.3% reduction in tumour volume relative to an untreated control group ($p < 0.05$) when the tumour was exposed to both external magnetic and ultrasound fields during administration of the MagMBs. In contrast, application of an external ultrasound field alone resulted in a 27% reduction in tumour volume. In addition, activated caspase and BAX protein levels were both observed to be significantly elevated in tumours harvested from animals treated with the MagMBs in the presence of magnetic and ultrasonic fields when compared to expression of those proteins in tumours from either the control or ultrasound field only groups ($p < 0.05$). These results suggest MagMBs have considerable potential as a platform to enable the targeted delivery of combined sonodynamic/antimetabolite therapy in pancreatic cancer.

## 1. Introduction

Pancreatic cancer has the lowest survival rate among the 21 most common forms of cancer with only 3% of patients surviving five years after their initial diagnosis [1]. While many other forms of cancer have seen survival rates increase significantly over the past four decades, the survival rate for pancreatic cancer has remained unchanged. Late presentation of patients due to the vague symptoms associated with the disease means only ~20% are eligible for potentially curative resection at the time of initial diagnosis [2]. Of the remaining ~80% of patients, ~50% present with metastatic disease and ~30% with locally advanced or borderline resectable pancreatic cancer (LAPC or BRPC) [3]. While earlier diagnosis and better awareness are key components of any future strategy to improve survival rates, there is also an urgent need for improved therapies. Several studies have investigated the potential of neo-adjuvant chemo- and/or radio-therapy to downstage tumours and increase the number of patients eligible for resection [3–5]. Unfortunately, such treatments are often associated with significant off-target effects due to the non-specific nature of the chemotherapy regimen. Therefore, the development of targeted treatments that reduce side-effects related to systemic chemotherapy have enormous potential as neo-adjuvant and palliative pancreatic cancer

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http://dx.doi.org/10.1016/j.jconrel.2017.07.040
Received 2 June 2017; Received in revised form 19 July 2017; Accepted 28 July 2017
Available online 29 July 2017

treatments by reducing tumour burden to either enable surgery or to provide symptom relief.

In a previous study, we demonstrated the utility of ultrasound responsive microbubbles (MBs) for delivery of drug payloads and encapsulated oxygen gas to pancreatic tumours [6]. MBs are lipid or polymer stabilised gas filled particles approved for use as contrast agents in diagnostic ultrasound [7]. At low ultrasound pressures, MBs oscillate in a relatively symmetric manner resulting in acoustic backscatter that enhances the quality of the diagnostic image [8]. Exposure of cells to low intensity ultrasound can also facilitate a phenomenon known as sonoporation which causes a transient 'poration' of cellular plasma membranes and the phenomenon is enhanced in the presence of exogenously-added MBs. Such an approach has been exploited to enhance the efficacy of gemcitabine therapy in pancreatic cancer patients [9,10]. In contrast, at higher acoustic pressures, collapse of the MB leads to rupture and release of the shell fragments at the target site [11]. This feature has been exploited by several groups investigating the potential of MBs as targeted delivery vehicles [12,13]. In our previous work, we attached the antimetabolite drug 5-fluouracil (5-FU) and the sonosensitiser Rose Bengal (RB) to the shell of oxygen-loaded lipid stabilised MBs for the combined antimetabolite and sonodynamic therapy (SDT) treatment of pancreatic cancer [6]. Significant reductions in the viability of three pancreatic cancer cell lines (BxPC3, MiaPaCa-2 and Panc-01) and inhibition of the growth of ectopic pancreatic BxPC-3 tumours were observed for the combined treatment when compared to either treatment alone. Antimetabolite therapy is an established treatment protocol for pancreatic cancer with 5-FU and gemcitabine among the most commonly used antimetabolite drugs [14]. In contrast, SDT is an emerging anti-cancer treatment that involves the activation of an otherwise inactive sensitiser drug using low-intensity ultrasound [15]. The combination of sensitiser and ultrasound, in the presence of molecular oxygen, generates cytotoxic levels of reactive oxygen species (ROS) causing cell death via oxidative stress [16]. As oxygen is a key substrate for the generation of ROS in SDT, and since pancreatic adenocarcinoma is characterised as extremely hypoxic, providing oxygen during SDT can improve the ROS yield and enhance the therapeutic outcome [17]. While our oxygen carrying MBs have shown great promise as a platform for targeted oxygen delivery and enhanced 5-FU/SDT treatment of pancreatic cancer, there remains a need to demonstrate the effectiveness of this method in an orthotopic tumour model following intravenous injection of the MB suspension. To this end, we have reasoned that an additional layer of targeting may be required to help retain MBs in the tumour vasculature after injection and enhance the quantity of MBs destroyed at the target site by ultrasound exposure. The incorporation of magnetic nanoparticles within the MB shell is one approach that has been explored to improve the targeting capability of MBs [18]. Previous work in our laboratory has demonstrated that externally applied magnetic fields may be used to enhance the retention of magnetically-responsive microbubbles at a target site in an ex vivo model under physiologically-relevant flow rates [19]. In this manuscript, we assess the ability of oxygen loaded magnetic MBs with 5-FU and Rose Bengal attached to their surface, as a targeted treatment for orthotopic human pancreatic BxPC-3 tumours in SCID mice. The benefit afforded by incorporating magnetic targeting into our delivery platform is demonstrated by studies in a flow-phantom and by therapeutic efficacy studies in vivo.

## 2. Materials and methods

### 2.1. Reagents and equipment

1,2-dibehenoyl-*sn*-glycero-3-phosphocholine (DBPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG(2000)) and DSPE-PEG(2000)-biotin were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Oxygen gas was purchased from BOC Industrial Gases UK and perfluorobutane (PFB) was purchased from Apollo Scientific Ltd. Phosphate Buffered Saline (PBS) was purchased from Gibco, Life Technologies, UK. Glycerol and propylene glycol (1 kg, hydrolysed) were purchased from Sigma Aldrich (UK). Superparamagnetic iron oxide nanoparticles (SPION): fluidMAG-Lipid (50 nm hydrodynamic diameter) were purchased from Chemicell (Berlin, Germany). The use of lipid conjugated SPION in this study was preferred over the use of previously reported isoparaffin stabilised SPION as the addition of lipids to lipid-shelled microbubbles is likely to be less disruptive to the acoustic response of the system compared to the addition of isoparaffin [19]. These microbubbles have been extensively characterised and successfully used in previous in vivo experiments [20]. The method for magnetic microbubble fabrication used in this study has then been adapted for the use of lipid conjugated SPION: fluidMAG-Lipid as presented in the following section. MBs were formed using a Microson ultrasonic cell disruptor, 100 W, 22.5 kHz, from Misonix Inc. (NY, USA). Optical microscope images were obtained using a Leica DM500 optical microscope. MB concentration and size were determined using purpose- written MATLAB software (2010B, MathWorks, Natick, MA, USA). Rose Bengal sodium salt, NHS-biotin, MTT assay kit, avidin, chloroacetic acid, 4-dimethylaminopyridine (DMAP), hydroxybenzotriazole (HOBt), *N*,*N*′-dicyclohexylcarbodiimide (DCC), anhydrous dimethylformamide (DMF), and ethanol were purchased from Sigma Aldrich (UK) at the highest grade possible. Biotin, 5-flurouracil, di(*N*-succinimidyl) carbonate and 2-aminoethanol were purchased from Tokyo Chemical Industry UK Ltd. Error was expressed as ± SEM (standard error of the mean) and statistical comparisons were established using ANOVA and un-paired Student's *t*-test.

### 2.2. Preparation of avidin functionalised magnetic microbubbles (MagPFBMBs)

Avidin functionalised magnetic MBs were prepared by dissolving DBPC (4.0 mg, 4.43 μmol), DSPE-PEG(2000) (1.35 mg, 0.481 μmol) and DSPE-PEG(2000)-biotin (1.45 mg, 0.481 μmol) at a molar ratio of 82:9:9 in chloroform (274 μL). The chloroform solvent was slowly evaporated by heating the lipid solution at 40 °C overnight to produce a dried lipid film. The lipid film was reconstituted in 2 mL of a PBS (pH 7.4 ± 0.1):propylene glycol:glycerol (8:1:1 v/v) mixture and the contents heated at 80 °C under stirring for 30 min in a water bath. FluidMAG-Lipids NPs (150 μL) were then added to the solution and the mixture was sonicated with a handheld sonicator probe for 1.5 min (100 W, 22.5 kHz, power setting 4). The headspace of the glass vial was then filled with perfluorobutane gas (PFB) and the gas/liquid interface was sonicated for 20 s (power setting 19), producing PFB-containing magnetic MBs (MagPFBMBs). The vial was immediately sealed and placed in an ice bath for 10 min. The MagPFBMB suspension was then centrifuged (100 RCF, 5 min) to remove the excess NPs and non-incorporated MB lipids by discarding the infranatant. The microbubble concentrate was re-suspended in 2 mL of PBS (pH 7.4 ± 0.1):propylene glycol:glycerol (8:1:1 v/v), avidin in PBS (50 μL, 10 mg/mL) was added to the suspension and the contents mixed for 10 min on a rotary shaker. The suspension was centrifuged (100 RCF, 5 min) to remove the excess avidin and the PFBMBs were again re-suspended in 2 mL of PBS (pH 7.4 ± 0.1):propylene glycol:glycerol (8:1:1 v/v). MagPFBMBs were analysed using a Leica DM500 optical microscope to obtain the size distribution and concentration. For this, 10 μL of suspension was diluted in 190 μL of PBS and examined using a haemocytometer (Bright-Line, Hausser Scientific, Horsham, PA, USA). 30 images were obtained with a 40 × objective lens and analysed with customised image analysis package in MATLAB (2010B, MathWorks, Natick, MA, USA). The iron content in the MagPFBMBs was determined by atomic absorption spectroscopy using a Varian fast sequential atomic absorption spectrometer. A calibration curve was constructed using known concentrations of Fe(III) in 0.5 M HCl. Readings were taken at 248.3 nm, 0.5 nm slit width, 10.0 mA lamp current, with the following flame settings; flame type: air/acetylene, air flow: 13.50 L/