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Oxygen generating nanoparticles for improved photodynamic therapy of hypoxic tumours

Yingjie Sheng[a], Heather Nesbitt[a], Bridgeen Callan[a], Mark A. Taylor[b], Mark Love[c], Anthony P. McHale[a,*], John F. Callan[a,*]

[a] Biomedical Sciences Research Institute, University of Ulster, Coleraine, Northern Ireland BT52 1SA, UK
[b] Department of HPB Surgery, Mater Hospital, Belfast, Northern Ireland BT14 6AB, UK
[c] Imaging Centre, The Royal Victoria Hospital, Grosvenor Road, Belfast, Northern Ireland BT12 6BA, UK

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ABSTRACT

Photodynamic therapy (PDT) is a clinically approved anti-cancer treatment that involves the activation of an otherwise inactive sensitiser drug with light, which in the presence of molecular oxygen, generates cytotoxic reactive oxygen species (ROS). As oxygen is a key requirement for the generation of ROS in PDT and given the fact that hypoxia is a characteristic of most solid cancerous tumours, treating hypoxic tumours using PDT can be a challenge. In this manuscript, we have prepared a CaO_2 nanoparticle (NP) formulation coated with a pH-sensitive polymer to enable the controlled generation of molecular oxygen as a function of pH. The polymer coat was designed to protect the particles from decomposition while in circulation but enable their activation at lower pH values in hypoxic regions of solid tumours. The oxygen generating capability of the polymer coated NPs was demonstrated in aqueous solution with minimal oxygen produced at pH 7.4, whereas it increased significantly when the pH was reduced to 6.2. The polymer coated CaO_2 NPs were also observed to significantly increase tumour pO_2 levels ($p < 0.05$) in mice bearing ectopic human xenograft MIA PaCa-2 pancreatic tumours with an average increase in tumour pO_2 of 6.5 mm Hg in the period 10–30 min following administration. A statistically significant improvement in PDT mediated efficacy ($p < 0.001$) was also observed when the particles were administered to mice bearing the same tumours 20 min prior to PDT treatment. These results suggest that the polymer coated CaO_2 NP formulation offers significant potential as an *in situ* method for oxygen generation to enhance the efficacy of treatments that depend on the presence of oxygen to elicit a cytotoxic effect.

1. Introduction

Photodynamic therapy (PDT) is a cancer treatment that involves irradiating a photoactive drug with light, in the presence of molecular oxygen, to generate toxic levels of reactive oxygen species (ROS) ultimately resulting in cell death [1]. By carefully controlling light delivery to the target lesion, ROS generation can be localised with high precision in three dimensions sparing healthy surrounding tissue. While the targeted nature of PDT remains its greatest attraction, the technique is significantly limited by the inability of light to penetrate deeply through human tissue [2]. This has restricted PDT to the treatment of superficial lesions and hindered its ability to treat larger solid tumours. The development of near-infrared (NIR) absorbing sensitisers and the emergence of sonodynamic therapy (SDT) promise to overcome this limitation by enabling the activation of sensitisers at greater depths *in vivo* [3]. Combatting hypoxia presents another challenge in the treatment of solid tumours using PDT/SDT as oxygen is a key requirement for the generation of ROS. This is particularly true for tumours of the pancreas [4] and hypoxia is now recognised as an indicator of poor prognosis for many types of cancer [5,6]. Inefficient gas and mass transfer resulting from atypical vascularisation together with elevated oxygen demand by hyper-proliferating tissues results in hypoxia in most solid tumours. Once a hypoxic environment develops in the tumour, cell populations become resistant to many conventional cancer chemotherapeutic agents through a variety of adaptive survival mechanisms. Similarly, for radiotherapy, oxygen has been shown to play a very significant role in enhancing radiation induced damage to nucleic acid in target tissues [7]. One of the major challenges associated with the latter has been to provide oxygen to the target tissues during therapy. A number of approaches have been employed including, hyperbaric oxygen breathing and breathing pure oxygen or carbogen at atmospheric pressure [8]. Such approaches, however, have delivered limited success and there is still a significant un-met need in this area. We have recently demonstrated that the selective destruction of oxygen loaded

* Corresponding authors.
E-mail addresses: ap.mchale@ulster.ac.uk (A.P. McHale), j.callan@ulster.ac.uk (J.F. Callan).

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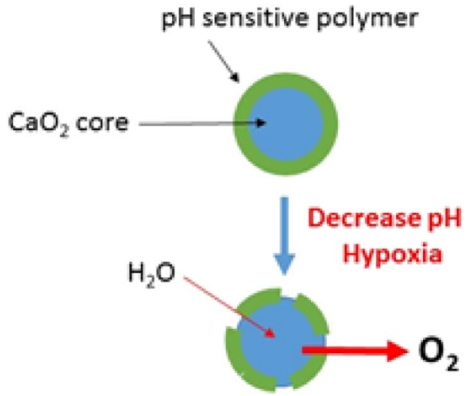

Scheme 1. Schematic illustration of how the polymer coat (green) protects the nanoparticle core (blue) from its aqueous environment at normal pH but at lower pH, the polymer coat dissolves, allowing access to the core by water with the resulting generation of oxygen.

microbubbles in the tumour microenvironment using low intensity ultrasound provided a temporary boost in tumour oxygen levels that enhanced the sonodynamic therapy (SDT) treatment of pancreatic tumours [9]. Inspired by these results, we are also focusing on the development of *in situ* oxygen generating nanoparticles as an alternative method to improve tumour oxygenation during PDT.

In this manuscript, we describe the preparation and characterisation of calcium peroxide (CaO_2) nanoparticles that generate molecular oxygen upon decomposition in water. The nanoparticles were coated with a pH-responsive methacrylate based *co*-polymer containing a tertiary amine residue that protects the nanoparticle core from water at pH values above 7.4. At lower pH values, the tertiary amine unit ionises resulting in dissolution of the polymer coat and exposure of the nanoparticle core to the aqueous environment resulting in oxygen generation (Scheme 1).

Tumour tissue interstitial fluid is more acidic (pH = ~6.0) than normal tissue, as hypoxia results in the accumulation of acid by inducing the production of energy from glycolysis *via* the Pasteur effect [10]. This difference in pH has been utilised in numerous pH-responsive cancer therapeutics and diagnostic probes [11]. Here, the pH-responsive polymer coat will limit NP decomposition in the blood and normal tissue while facilitating its decomposition in more acidic cancer tissue. The ability of the resulting oxygen generation to improve oxygenation in hypoxic environments and enhance the PDT-mediated treatment of BxPC-3 pancreatic cancer cells *in vitro* and human xenograft MIA-PaCa-2 pancreatic tumours *in vivo* is demonstrated.

2. Materials and methods

2.1. Reagents and equipment

Calcium chloride, PEG 200, 1 M ammonia solution, 35% hydrogen peroxide, sodium hydroxide, phosphate buffered saline (PBS), luminol, methanol, ethanol, hexane, chloroform, Rose Bengal (RB), singlet oxygen sensor green (SOSG), anhydrous tetrahydrofuran (THF), 2-(dimethylamino)ethyl methacrylate, methyl methacrylate, ethyl acrylate and 1,1′-Azobis(cyclohexanecarbonitrile) (ABCN) were purchased from commercial sources at the highest possible grade. BxPC-3 and MIA PaCa cells were obtained from the American Type Culture Collection (ATCC) and matrigel from BD Biosciences, Erembodegem, Belgium. SCID mice (C.B-17/IcrHanHsd-Prkdc^{SCID}) were bred in house. Scanning electron microscopy (SEM) analysis was conducted using an "FEI Quanta" scanning electron microscope while dynamic light scattering (DLS) measurements were performed using a Malvern Zetasizer 3000HSA (Malvern, Worcs., UK). Dissolved oxygen measurements were recorded using a Thermo Scientific™ DO Probe Orion™ 083005MD (Fisher Scientific, Ottawa, ON, Canada) while nanoparticle solutions were mixed using a Silverson homogenizer (Silverson Machines Ltd., Chesham, U.K.). Fluorescence measurements were undertaken using a Cary Eclipse spectrophotometer while 96 well plates were analysed using a Fluostar Omega plate reader. Tumour pO_2 measurements were performed using an Oxylite oxygen electrode sensor (Oxford Optronics, Oxford, UK). NMR spectra were obtained on Varian 500 MHz instrument at 25.0 ± 1 °C and processed using Bruker software. Mass spectra were obtained using a Finnegan LCQ-MS instrument. Error in measurements was expressed as % standard error of the mean while statistical analysis was undertaken using 2-tailed Students *t*-test.

2.2. Preparation of uncoated CaO_2 NPs

CaO_2 nanoparticles were prepared following the method described by Khodaveisi et al. [12] Ammonia solution (15.0 mL, 1 M) and PEG 200 (120.0 mL, 0.6744 mol) was added to a stirred solution of calcium chloride (3.0 g, 0.027 mol) in distilled water (30 mL). A solution of 35% H_2O_2 (15 mL, 0.17 mol) was then added to the mixture at a rate of 3 drops per minute and the colourless solution stirred for a further 2 h at room temperature. A NaOH solution (0.1 M) was then added until a pH value of 11.5 was achieved when the solution changed to a white coloured suspension. The precipitate was separated by centrifugation (8000 g, 5 min) and the resulting pellet washed three times with NaOH (25 mL, 0.1 M). The precipitate was then washed with distilled water until the filtrate pH reached 8.4 and the resulting solid dried *in vacuo* at 80 °C for 2 h. The resulting particles were suspended in ethanol and sonicated for 5 min. The suspension was passed through a Millex Filter Unit (0.45 μm) to isolate larger particles and the filtrate concentrated to dryness providing the uncoated CaO_2 nanoparticles as a white powder. The size of the nanoparticles was determined by SEM and DLS.

2.3. Determination of CaO_2 content in the uncoated CaO_2 NPs

The active CaO_2 content of the NPs was determined by reaction with luminol in PBS. A chemiluminescence/concentration calibration curve for the reaction of H_2O_2 with luminol was performed according to the procedure adopted by Komagoe et al. [13] CaO_2 NPs suspended in ethanol (50 μL, 35.6 μM) were added to a luminol solution (50 μL, 10 mg/mL in PBS) and the luminescence intensity determined using a plate reader. The CaO_2 content was determined by indirectly measuring the number of moles of H_2O_2 produced (by reference to a calibration graph) from the fixed mass of CaO_2 powder and assuming all the available CaO_2 was converted to H_2O_2.

2.4. Determination of singlet oxygen generation

The ability of the CaO_2 NPs to enhance PDT mediated singlet oxygen generation was determined using the singlet oxygen probe SOSG. CaO_2 NPs (2 mg) in de-oxygenated ethanol (1 mL) were added to a de-oxygenated PBS solution containing SOSG and RB resulting in final concentrations of 2.5 μM (SOSG), 5.0 μM (RB) and 35.6 μM (CaO_2 NPs). The solutions were then exposed to white light for 5 min (Fenix LD01 LED,50 mW output,113.0 J/cm^2) Control experiments were also undertaken and included (i) CaO_2 NP + light and (ii) RB + light. The intensity of SOSG fluorescence at 525 nm upon excitation at 505 nm was recorded at the beginning and at the end of each experiment.

2.5. In vitro PDT experiments

BxPc3 cells were seeded in a 96 well plate at a density of 5 × 10^4 cells per well and incubated in a hypoxic chamber at 37 °C ($O_2/CO_2/N_2$, 0.1: 5: 94.9, v/v/v) for 3 h. The cells were then treated with either (i) RB or (ii) RB with CaO_2 NPs to reach a final concentration per well of 1 μM (RB) and 25 μM (CaO_2) respectively. The CaO_2 NPs were initially prepared in EtOH that was diluted 1:1 v/v with PBS (100 μL total