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Note

Ultrasound-mediated oxygen delivery from chitosan nanobubbles

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ABSTRACT

Ultrasound (US) energy combined with gas-filled microbubbles has been used for several years in medical imaging. This study investigated the ability of oxygen-loaded chitosan bubbles to exchange oxygen in the presence or in the absence of US. Oxygen delivery is enhanced by sonication and both frequency and time duration of US affected the exchange kinetics.

Ultrasound (US) is best known for its imaging capability in diagnostic medicine (Nelson and Pretorius, 1998). Several researchers groups have studied the use of diagnostic US (0.5–5 MHz) with or without microbubbles for local drug delivery which upon accumulation in required areas can be made leaky by the locally applied external ultrasound, and can liberate incorporated drugs (Torchilin, 2009; Liu et al., 2006; Unger et al., 2002; Rapoport et al., 2004).

This note proposes two formulations (A and B) of oxygen-filled nano- and microbubbles for therapeutic application in hypoxic conditions. Local hypoxia is related to various serious diseases, from inflammatory conditions to cancerous lesions. Tumor hypoxia is a therapeutic problem because of its adverse impact on the effectiveness of radiotherapy and chemotherapy (Vaupel and Harrison, 2004; Zhou et al., 2006).

The two formulations, coated with chitosan were prepared and characterized, and their ability to release oxygen in the presence or otherwise of ultrasound was studied at 25 °C. The nanobubbles' efficacy was investigated by determining their effect on hypoxia inducible factor-1α (HIF-1α) on JEG-3 human choriocarcinoma cells. The formulations were prepared as follows.

Formulation A: a NaCl aqueous solution (0.9% w/w, pH 5.0) and a palmitic acid (0.3% w/v) ethanol solution were introduced into a three-necked round-bottom flask and a 2.7% chitosan solution in acetate buffer pH 5.0 was added dropwise under stirring. The mixture was saturated with oxygen using a gas purge, monitoring the oxygen concentration with an oxymeter (Portamess 913 OXY) up to 35 mg/l. A high-shear mixer (Ultraturrax) was used for 2 min while continuing the O_2 purge. To stabilize the chitosan shell, a β-glycerol phosphate (50 mg/ml) and a 1N NaOH solution were added, to pH 6.5.

Formulation B was prepared likewise, but perfluoropentane was added to increase oxygen loading, since oxygen can be stored in perfluorocarbons (Van Liew and Burkard, 1996; Burkard and Van Liew, 1994). The compositions of the two formulations are in Table 1.

To characterize the nano/microbubbles, the average diameter, polydispersity index and zeta potential were determined at 25 °C using a 90 Plus instrument (Brookhaven). The formulations were also evaluated by optical and electronic microscopy to verify size and shape. All experiments were performed in triplicate.

The formulation stability, when stored at 25 °C, was evaluated over time for up to 24 h, determining size and shape of the nanobubbles by photon correlation spectroscopy (PCS) and transmission electron microscopy (TEM), respectively.

This note focuses on oxygen release from the formulations, evaluated in the presence or otherwise of US in simulated hypoxic conditions. To determine O_2 release, 3 ml of each formulation (O_2 concentration about 30 mg/l) were injected into 20 ml of hypoxic (O_2 concentration 0.4 mg/l) saline solution (0.9% mg/ml). The oxygen concentration was monitored in the saline solution at 25 °C for 60 min to detect gas release.

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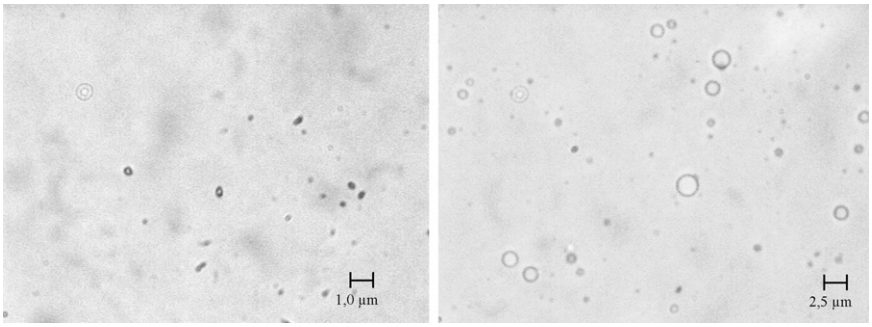

Fig. 1. Optical microphotographs of formulations A and B.

Table 1
Composition of formulations A and B.

Components	A (% w/v)	B (% w/v)
Sodium chloride	0.61	–
Palmitic acid	0.02	0.02
Epikuron® 200	–	0.05
Chitosan (medium Mw)	0.20	0.16
Perfluoropentane	–	7.03
β-Glicerolphosphate	0.34	–
NaOH	0.01	–
Water	95.36	88.94
Ethanol	3.46	3.80

To evaluate O_2 release in the presence of US, 10 ml of oxygen-filled formulations were injected into a small plastic container and dipped into a thermostatic water bath to provide US propagation. The oxygen present in the solutions containing the gas-filled nano/microbubbles was measured after 2, 5 or 10 min sonication using US having frequency = 45 kHz, 260 W peak power.

To determine stability of formulations in the presence of US, morphological analysis and average diameter were determined after 2, 5, and 10 min sonication (Oeffinger and Wheatley, 2004).

The hemolytic activity of the two formulations was evaluated on human blood. Different percentages v/v (1.7, 3.3, 6.7, 10, and 13.3%) of nano/microbubbles were added to an erythrocyte suspension (30%, v/v) in PBS. After 90 min of incubation at 37 °C, the samples were centrifuged and the supernatants were analyzed spectrophotometrically. The cytotoxicity and the effect of nanobubbles on HIF-1α was then investigated on JEG3 cell line.

Table 2 shows the physico-chemical characteristics of the two formulations. The positive values of the zeta potential confirm the presence of chitosan on the surface (Hoven et al., 2006).

Oxygen-filled nanobubbles in formulation A had a diameter of about 700 nm, while in formulation B the diameter was about 1.2 μm. Fig. 1 shows the narrow size distribution and spherical morphology.

Table 2
Physico-chemical characteristics of the two formulations ($n = 3$).

Formulation	Diameter ± SD (nm)	Polydisp. index	PZ ± SD	pH
A	708 ± 51.3	0.23	12.07 ± 1.33	6.50
B	1236.5 ± 17.5	0.25	26.87 ± 1.61	5.20

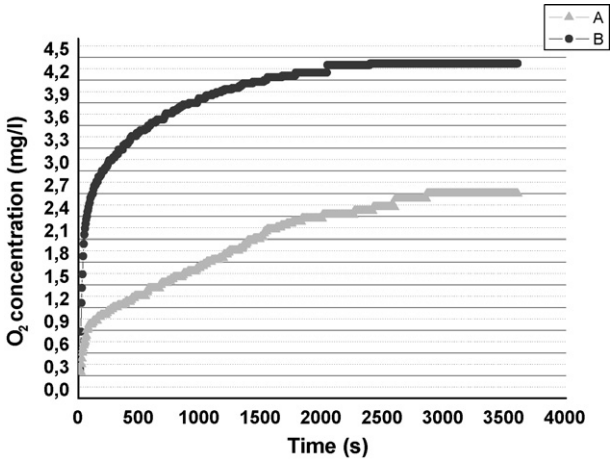

Fig. 2. Profiles of oxygen release from the two formulations over time.

In both formulations there was a well-defined shell of about 30 nm, which might stabilize the bubbles. The bubbles containing perfluorocarbon gas showed a better well-defined core–shell structure.

The spherical shape and constant average diameter of the formulations 24 h after their preparation confirmed their stability (data not shown). Formulations A and B both increased the oxygen concentration of hypoxic solutions. Fig. 2 shows the oxygen release profiles from the two formulations, starting from hypoxic condi-

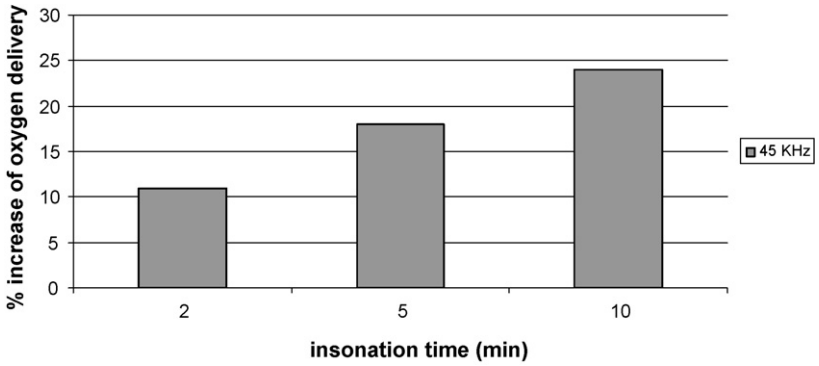

Fig. 3. Effect of US on O_2 release from formulation A after 2, 5 and 10 min.

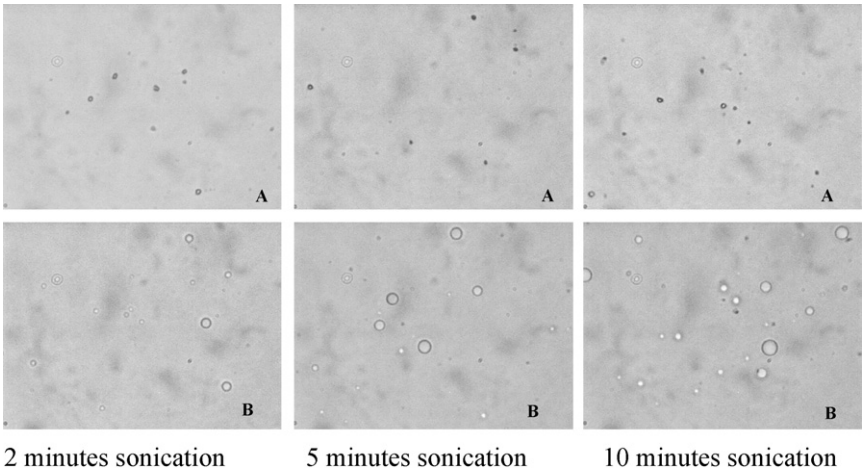

Fig. 4. Morphology of formulations A and B after exposure to 2, 5 and 10 min ultrasound.

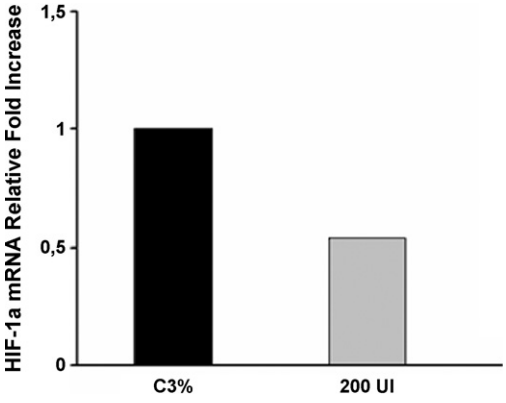

Fig. 5. HIF-1α expression after incubation of formulation A.

tions (0.4 mg/l); after 60 min, formulation B had delivered more oxygen (4.6 mg/l) than formulation A (2.7 mg/l). Formulation B can also store more oxygen, due to the perfluorocarbon core. This ability of chitosan nano/microbubbles makes them a promising tool for oxygen delivery in hypoxic environments.

To enhance oxygen release US sonication was applied and was found to potentiate oxygen delivery to the hypoxic solution, increasing O_2 release by about 20% after 10 min (Fig. 3). This enhancement might be related to the increased permeability of the chitosan shell. Optical analysis (Fig. 4) and size determination by PCS showed that US does not affect the structure of the formulations.

The increased O_2 delivery is proportional to sonication time, and thus it may be hypothesized that if sonication time is shorter, release is slower.

The lactate dehydrogenase (LDH) assay (Biovision) revealed that chitosan formulations had no cytotoxic effect on JEG-3 choriocarcinoma cells. Preliminary investigations evaluated the efficacy of these new oxygen delivery systems. The effect of formulation A was studied on HIF-1α, the major player in the physiological response to hypoxia (Zhou et al., 2006). Our exploratory data on human JEG-3 cells showed a reduction of HIF-1α expression, versus untreated control cells, when cells were incubated in hypoxic conditions (at 3% pO_2) and treated with 200 μl of O_2-filled nanobubbles (Fig. 5).

To conclude, this research shows that oxygen-filled nano/microbubbles coated with chitosan exchange oxygen with the surrounding hypoxic solution. The oxygen release is enhanced by sonicating the particles with US at a frequency of 45 kHz.

Acknowledgements

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