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An Implantable Depot That Can Generate Oxygen in Situ for Overcoming Hypoxia-Induced Resistance to Anticancer Drugs in Chemotherapy

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Supporting Information

ABSTRACT: In the absence of adequate oxygen, cancer cells that are grown in hypoxic solid tumors resist treatment using antitumor drugs (such as doxorubicin, DOX), owing to their attenuated intracellular production of reactive oxygen species (ROS). Hyperbaric oxygen (HBO) therapy favorably improves oxygen transport to the hypoxic tumor tissues, thereby increasing the sensitivity of tumor cells to DOX. However, the use of HBO with DOX potentiates the ROS-mediated cytotoxicity of the drug toward normal tissues. In this work, we hypothesize that regional oxygen treatment by an implanted oxygen-generating depot may enhance the cytotoxicity of DOX against malignant tissues in a highly site-specific manner, without raising systemic oxygen levels. Upon implantation close to the tumor, the oxygen-generating depot reacts with the interstitial medium to produce oxygen in situ, effectively shrinking the hypoxic regions in the tumor tissues. Increasing the local availability of oxygen causes the cytotoxicity of DOX that is accumulated in the tumors to be significantly enhanced by the elevated production of ROS, ultimately allaying the hypoxia-induced DOX resistance in solid malignancies. Importantly, this enhancement of cytotoxicity is limited to the site of the tumors, and this feature of the system that is proposed herein is unique.

Chemotherapy that involves the use of antitumor drugs such as doxorubicin (DOX) has been regarded as critical in the treatment of a range of human cancers.[1] In an aerobic environment, the intracellular metabolite of DOX reacts with oxygen and produces reactive oxygen species (ROS), which oxidize and damage major cellular components, ultimately resulting in the death of cancer cells.[2] However, the effectiveness of DOX is considerably limited in a hypoxic microenvironment ($pO_2 \leq 2.5$ mmHg),[3] which is typically present in the central region of solid tumors, such as glioblastoma multiforme, cervical cancer, lung adenocarcinoma, and hepatocellular carcinoma,[3−5] owing to their poorly formed vasculature and low vascular density.[6] Recent investigations have established that in the absence of an adequate concentration of oxygen, tumor cells that are grown in the hypoxic region are resistant to DOX treatment, owing to their attenuated intracellular generation of ROS.[4,7]

One of the proposed methods for strengthening the cytotoxic effect of DOX on tumor cells is to increase the supply of oxygen to cancerous tissues.[3,7] Hyperbaric oxygen (HBO) therapy, in which the patient is administered pure oxygen in a pressurized chamber, favorably raises the oxygen partial pressure in plasma, promoting oxygen transport to the hypoxic tumor tissues.[8] Alleviation of hypoxia in tumor tissues enables oxygen to augment the sensitivity of tumor cells to DOX because of their excessive production of ROS,[6] inhibiting tumor progression; this method is particularly useful for nonresectable cancers.[9] However, the use of HBO together with DOX may potentiate the ROS-mediated cytotoxicity of the drug toward normal tissues.[5,7] Hence, systemically administered antitumor medication with DOX is considered to be an absolute contraindication for HBO therapy.[5,10]

In this work, we hypothesize that regional oxygen treatment provided using an implantable oxygen-generating depot may improve the cytotoxicity of DOX to fight against malignant tissues in a highly site-specific manner, without increasing systemic oxygen levels. This oxygen-generating depot is prepared by dropping an alginate solution that contains calcium peroxide (CaO_2) and catalase into a calcium chloride ($CaCl_2$) bath to form the Ca^{2+}-cross-linked microencapsulated pellets, which are harvested by filtration and then air-dried. The use of alginate for microencapsulation generally involves the dispersion of droplets of alginate/payload into a gelation medium that contains divalent cations such as Ca^{2+}.[11] Alginate, which is an anionic polymer with a pK_a value of approximately 3.8, is ionized in deionized water (ca. pH 6.0), so its guluronic acid residues can interact with Ca^{2+} ions for which they have a high affinity to form the "egg-box" structure of physically cross-linked hydrogels.[11]

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Figure 1a schematically displays the structure of the as-prepared oxygen-generating alginate pellets and the mechanism

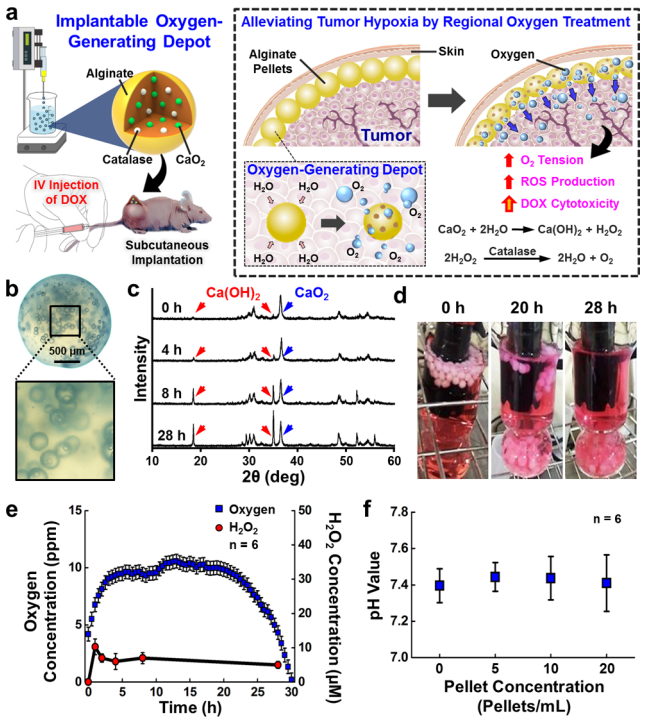

Figure 1. (a) Structure/composition of alginate pellets that act as an implantable depot for intratumoral delivery of oxygen and their mechanism of enhancement of cytotoxic effect of DOX on tumor cells. (b) Photomicrograph of generation of oxygen in test pellet following exposure to culture medium. (c) X-ray diffraction patterns that reveal decomposition of encapsulated CaO_2 and formation of $Ca(OH)_2$. (d) Photographs that show floating/settling behavior of oxygen-generating alginate pellets in culture medium. (e) Generation of O_2 and H_2O_2 of alginate pellets in culture medium. (f) Variation of pH of culture medium following treatment with various doses of alginate pellets.

of their operation. Upon implantation close to the tumor, the CaO_2 that is encapsulated in the alginate pellets reacts with the water that infiltrates the pellets from the interstitial tissues to produce calcium hydroxide [$Ca(OH)_2$] and hydrogen peroxide (H_2O_2), which is then spontaneously decomposed to form oxygen.[12,13] However, H_2O_2 is relatively stable, decomposing only very slowly in the absence of a catalyst. To eliminate this concern, catalase, a critical antioxidant enzyme in the removal of H_2O_2, is included in the alginate pellets to catalyze the breakdown of H_2O_2 into oxygen molecules.[12] The Ca^{2+}-cross-linked polymeric alginate matrix can temper the hydrolytic reactivity of CaO_2/catalase by limiting the infiltration of water molecules into the implanted pellets, prolonging the generation of oxygen and thereby increasing the efficacy of DOX treatment.[13]

The as-prepared alginate pellets had a diameter of 1.8 ± 0.1 mm (n = 6 batches), as determined using the Image-Pro Plus software from photographs taken under an optical microscope. Upon exposure to water, the rapid formation of gas bubbles was clearly observed within the pellets (Figure 1b), caused by the hydrolytic activation of CaO_2 in the presence of catalase to produce oxygen. As CaO_2 decomposes, along with O_2, $Ca(OH)_2$ is formed together with the intermediate product H_2O_2. In X-ray diffraction analysis, the intensity of the diffraction peak of CaO_2 declined over time, while that of $Ca(OH)_2$ increased steadily (Figure 1c). The oxygen-generating pellets freely floated in the test medium and then settled gradually (Figure 1d). The generation of oxygen from the test pellets lasted for a period of approximately 28 h (Figure 1e), while the accumulation of H_2O_2 was minimal throughout the experiment. Furthermore, the pH of the medium, which contained various concentrations of alginate pellets, remained neutral (pH 7.4, Figure 1f), suggesting that the formed hydroxide ions ($Ca(OH)_2$) did not alter the buffering capacity of the test medium.

The impact of hypoxia on the cellular uptake of DOX and its subsequent induction of cytotoxicity are studied using Hep3B tumor cells (which constitute a human hepatocellular carcinoma cell line). The percentage of cells that internalized DOX and their fluorescence intensity were quantified by flow cytometry, while the viability of cells that were cultured in the media that contained various concentrations of DOX under hypoxic (1% O_2) or normoxic (21% O_2) conditions was evaluated using the MTT assay. According to Figure 2a, the

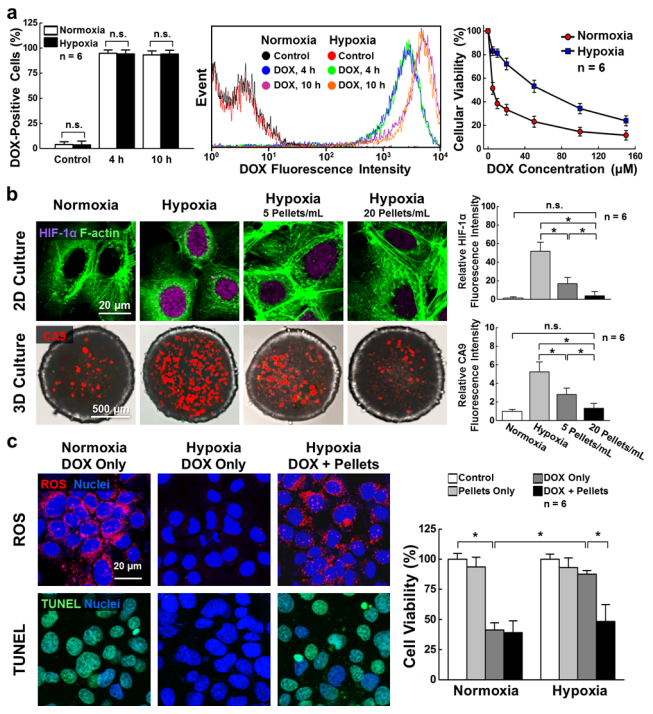

Figure 2. (a) Percentage uptake by, and fluorescent intensity of, Hep3B cells following incubation with DOX and their induced cytotoxicity under normoxic/hypoxic conditions. (b) Fluorescence images and corresponding fluorescence intensities of hypoxic markers HIF-1α and CA9 detected in cells in 2D and 3D cultures, respectively, under normoxic/hypoxic conditions. (c) CLSM images of intracellular production of ROS in test cells and corresponding images of TUNEL staining and results of MTT assay following various treatments. *P < 0.05; n.s.: not significant.

percentage of DOX-positive cells and their corresponding fluorescence intensity in the hypoxic culture were similar to those in the normoxic culture (P > 0.05). However, the MTT_{50} value of DOX toward hypoxic cells (60.2 μM) significantly exceeded that toward normoxic cells (7.1 μM, P < 0.05), suggesting that hypoxic cells were more resistant to DOX treatment than were normoxic cells. Numerous anticancer drugs, including DOX, have been reported to exhibit oxygen-dependent cytotoxicity.[3]

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The cytotoxicity of the as-prepared alginate pellets was qualitatively studied using a live/dead staining method and quantitatively measured by the MTT assay. In live/dead staining, live cells appear green, while the nuclei of dead cells appear red. According to Figure S1, no cytotoxicity toward the test cells was detected up to a concentration of 20 pellets/mL. The relief of hypoxia by alginate pellets in Hep3B tumor cells that were cultured in 2D or 3D formats was then examined in vitro. Three-dimensional aggregates of 50,000 Hep3B cells, with a diameter of around 1.2 mm, were constructed in wells that contained the methylcellulose hydrogel system, as described elsewhere.[14]

The incubation of cells under low oxygen tension has been shown to cause the intracellular accumulation of hypoxia-inducible factor (HIF)-1α, which subsequently promotes the upregulation of carbonic anhydrase 9 (CA9).[15] Therefore, both HIF-1α and CA9 have been frequently used as endogenous markers of hypoxia. As presented in Figure 2b, under normally oxygenated conditions (21% O_2), no apparent accumulation of HIF-1α was detected in cells in the 2D culture, whereas some cells in the interior of the 3D cell aggregate expressed a low level of the hypoxic marker CA9. Cell aggregates with a radius that exceeds the limit of oxygen diffusion capacity (typically 150−250 μm) are likely to develop a hypoxic microenvironment in their inner cores.[16] Following exposure to low oxygen tension (1% O_2), the Hep3B cells in the 2D culture exhibited strong HIF-1α staining, and almost the entire cell aggregate in the 3D culture became hypoxic with a high level of CA9 expression.

When the oxygen-generating alginate pellets were used, a dose-dependent reduction in the fluorescence intensity of HIF-1α (CA9) was clearly observed in Hep3B cells in the 2D (3D) culture ($P < 0.05$). The presence of 20 pellets/mL eliminated a significant proportion of hypoxia markers that were induced in the cells that were cultured in either 2D or 3D format under low oxygen tension, and this proportion was close to that in control cells that were cultured under standard oxygen tension ($P > 0.05$), suggesting that the alginate pellets that were prepared herein may serve as an in situ oxygen-generating reservoir in relieving hypoxia in solid tumors.

The capacity of the as-prepared alginate pellets to increase the ROS-mediated cytotoxicity of DOX against Hep3B tumor cells under normoxic or hypoxic conditions was investigated. The untreated cells and those that received DOX or had been treated with pellets alone served as the control groups. The production of cellular ROS that was induced by DOX treatment was detected by CellROX Deep Red, which is a ROS-sensitive dye.[17] Cells that underwent apoptosis were identified by TUNEL staining, and the viability of the cells was further studied by the MTT assay.

In normoxic culture, most of the Hep3B cells that were treated with DOX exhibited an elevated level of ROS and an increased incidence of apoptosis, and ultimately a reduced cell viability ($P < 0.05$, Figures 2c and S2). Under low oxygen tension, however, the ability of DOX to induce ROS production was substantially reduced, rendering the hypoxic cells more resistant to DOX treatment ($P < 0.05$). Notably, treatment with the oxygen-generating pellets considerably improved the accessibility of oxygen molecules to the hypoxic cells, potentiating the ROS-induced cytotoxic effect of DOX ($P < 0.05$). These empirical results demonstrate that the local provision of oxygen by the alginate pellets increases the sensitivity of cancer cells to DOX treatment.

On account of the promising in vitro results, the ability of the oxygen-generating pellets to reduce tumor hypoxia was examined in a nude mouse model. To produce the animal model, Hep3B cells were implanted subcutaneously in the right flank region of mice. When the tumors had grown to a volume of 150−200 mm^3, various concentrations of test pellets were implanted subcutaneously close to the tumors. To detect the tumor hypoxia, a fluorescent imaging agent, HypoxiSense 680, that has a high specificity for the hypoxic marker CA9, was administered via the tail vein. Twenty-four hours later, the mice were sacrificed, and their tumors were harvested and observed using an in vivo imaging system (IVIS).

According to Figure 3a,b, strong CA9 fluorescence emission was detected in the interior of the tumor in the untreated

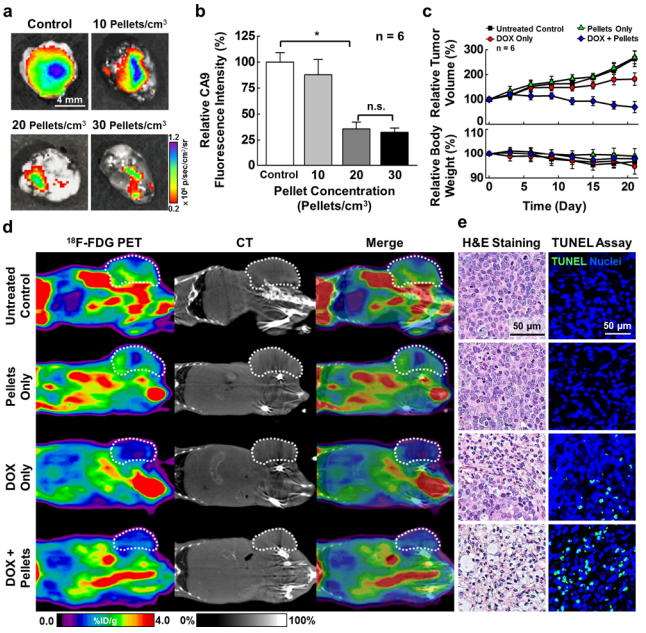

Figure 3. (a) Fluorescence images of hypoxic marker CA9 and (b) corresponding fluorescence intensities of tumors in mice after treatment with various concentrations of alginate pellets. (c) Changes in relative tumor volume and body weight of mice with Hep3B tumors in response to various treatments. (d) Antitumor efficacy of each treatment modality, as revealed by images of PET and (e) H&E and TUNEL staining. Dashed lines outline the tumor border. *$P < 0.05$; n.s.: not significant.

control group, revealing that the solid malignancy incorporated a central hypoxic region. In contrast, the implantation of oxygen-generating pellets significantly reduced the CA9 fluorescence intensity of the hypoxic tumor, indicating that the alginate pellets that were developed herein served as an implantable depot for the intratumoral delivery of oxygen to relieve tissue hypoxia (Figure 1a). The CA9 fluorescence intensity of hypoxia reached its lowest value at a dose of 20 pellets/cm^3; no further decline in fluorescence intensity was detected when a higher dose of pellets was applied, probably owing to the limitation on the amount of the available interstitial medium that could infiltrate the implanted pellets.[3] Therefore, a dose of 20 pellets/cm^3 was used in combination with the conventional chemotherapeutic agent DOX in the subsequent antitumor study.

Finally, the effectiveness of the oxygen-delivery depot in increasing the chemotherapeutic effect of DOX was evaluated

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in the tumor-bearing mice. The mice were subcutaneously implanted with test pellets, and 24 h later, DOX was administered via the tail vein. This process was repeated every 7 days for a total of three treatment sessions. The untreated mice (untreated control) and the mice that had received DOX or alginate pellets alone were the controls.

A comparison with the untreated control revealed no apparent reduction of the tumor volume in the group that had received only alginate pellets ($P > 0.05$, Figure 3c), whereas the group that had been treated with DOX alone exhibited inadequate antitumor activity. In marked contrast, tumor progression was effectively inhibited in the animals that received pellets + DOX, while their body weight loss was very small throughout the entire study. This encouraging result most likely follows from the fact that the cytotoxicity of DOX that accumulated in the tumors was greatly increased by the implanted oxygen-generating depot, effectively inducing the production of excess ROS (Figure 2c). Whereas HBO therapy enhances the oxygen-derived free radical-mediated toxicity of DOX to other normal tissues,[8] animals that received pellets + DOX exhibited increased cytotoxicity only at the sites of tumors.

At the end of repeated treatments, the improvement of the cytotoxicity of DOX against tumors that had been induced by the oxygen-delivery depot was further assessed by positron emission tomography (PET). ^{18}F-fludeoxyglucose (^{18}F-FDG) is a radiolabeled glucose analogue that has been widely utilized as a PET imaging agent for diagnosing cancer. Enhanced ^{18}F-FDG uptake typically correlates with high metabolic/proliferative activity of tumor tissues.[18] According to Figure 3d, the maximum uptake of ^{18}F-FDG in mice that were treated with pellets + DOX (1.68 ± 0.06%ID/g) was significantly lower than that in untreated mice (untreated control, 3.32 ± 0.16% ID/g) or in those that were treated with DOX (2.36 ± 0.33% ID/g) or alginate pellets (3.25 ± 0.14%ID/g) alone, revealing a drastic reduction in metabolic/proliferative activity of the tumor cells ($P < 0.05$).

To gain insight into the cytotoxic effect of DOX following treatment, the tumor tissues were retrieved and processed for histological analyses. As presented in Figure 3e, combining pellet implantation with DOX administration substantially reduced the number of tumor cells (hematoxylin−eosin, H&E, stain) and increased the population of apoptotic cells (TUNEL-positive). These empirical data reveal that the combinational strategy that involved locally implanted oxygen-generating pellets and systemically administered DOX is the most efficient method among all of the investigated methods for inducing the apoptosis of tumor tissues.

In summary, the implantable alginate pellets that were prepared in the study served as an in situ oxygen-generating depot that effectively alleviated the hypoxic regions in tumor tissues. Increasing the local availability of oxygen significantly increased the chemotherapeutic effect of DOX that was accumulated in the tumors by promoting ROS production. These analytical results demonstrate the feasibility of using this oxygen-generating system to potentiate the cytotoxicity of DOX in a highly site-specific manner, serving to allay the hypoxia-induced DOX resistance in solid malignancies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b01784.

Experimental details, additional data and figures (PDF)

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Notes

The authors declare no competing financial interest.

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