Hypoxia, a characteristic hallmark of solid tumours, restricts the therapeutic effect of photodynamic therapy (PDT) for cancer treatment. To address this issue, a facile and nanosized oxygen (O₂) bubble template is established by mixing oxygenated water and water-soluble solvents for guiding hollow polydopamine (HPDA) synthesis, and O₂ is encapsulated in the cavity of HPDA. HPDA with abundant catechol is designed as a carrier for zinc phthalocyanine (ZnPc, a boronic acid modiﬁed photosensitizer) via borate ester bonds to fabricate nanomedicine (denoted as HZNPs). The in vitro and in vivo results indicate that O₂-evolving HZNPs could alleviate tumour hypoxia and enhance PDT-anticancer efficiency. Melanin-like HPDA with a photothermal conversion rate (η) of 38.2% shows excellent synergistic photothermal therapy (PTT) efficiency in cancer treatment.

Introduction

Malignant cellular proliferation and vascular malformation lead to hypoxia formation in solid tumours.1,2 Insufficient oxygen (O₂) supply and consumption induce the invasion and metastasis of tumour cells.3,4 Hypoxia severely limits the treatment outcome of O₂-mediated cancer therapies, especially photodynamic therapy (PDT).5

Great efforts have been made to alleviate tumour hypoxia for enhancing drug therapy efﬁciency, and these types can be divided into two categories, supplying exogenous O₂ to tumours (carrier or carrier-free) and catalysing endogenous hydrogen peroxide (H₂O₂) or H₂O to generate O₂.6–8 Delivering exogenous O₂ to alleviate tumour hypoxia is the most direct and effective method, and hyperbaric O₂ therapy increasing the pO₂ in tumor tissues has been used in clinical PDT for cancer treatment.9 In recent years, haemoglobin (Hb), liposomes and macrovesicle materials were designed as carriers for O₂ delivery. Hb, an iron-rich metalloprotein in red blood cells, could highly bind and deliver O₂ in intravascular transmission.10 However, free Hb is susceptible to auto-oxidation during circulation and results in renal toxicity and cardiovascular complications.11 Liposomes embedded with microbubbles have been approved by the FDA as an ultrasound contrast agent for echocardiography.12 Its disadvantage is that the O₂ loading efﬁciency is approximately 10% by volume, indicating that it is sufﬁcient for delivering potent bioactive gases, such as NO and Xe, but not suitable for O₂.13 Compared with liposomes, hollow materials have a core and a high loading efﬁciency for O₂. How to fabricate a satisfactory O₂ carrier to alleviate tumour hypoxia is still a big challenge.

Polydopamine (PDA) is an ideal carrier for drug delivery because of its good biocompatibility.14 PDA formation shares many common characteristics with the melanin synthetic pathway, and its photothermal conversion rate (η) could reach up to 40%.15 Under alkaline conditions, dopamine (DA) monomers were oxidized to DA quinone and a ﬁve-membered ring, then rearranged to 5,6-dihydroxyindoles and aggregated with other polymers.16 More importantly, the polymerization rate of DA at the gas–liquid interface is higher than that in the solutions,17,18 indicating that the O₂ bubble template may guide PDA polymerization. Chen’s group reported a method for O₂-rich PDA microcapsule preparation in 2021, but the micron-sized aggregates limited its further application in cancer treatment.19
As shown in Scheme 1, a facile and nanosized O2 bubble template is reported by mixing oxygenated water and oxygenated water-soluble organic solvents. The O2 bubble template guided DA polymerization to form hollow PDA (HPDA) and encapsulate O2 simultaneously. Furthermore, HPDA is designed as a carrier for zinc phthalocyanine (ZnPc) to fabricate pH-responsive nanomedicine (denoted HZNPs). The ZnPc release rate of HZNPs reached 64.9% in a simulated tumour microenvironment. The in vitro and in vivo results indicated that HZNPs could deliver O2 to alleviate tumour hypoxia, inhibit the expression of hypoxia-induced factor-1 (HIF-1α), and enhance the anticancer efficiency of PDT. Upon the combination of light irradiation (808 nm + 665 nm), HZNP treated groups achieved satisfactory outcomes by PTT and PDT.

Results and discussion

Gas solubility in solvents is closely related to polarity, and mixing different solvents could change gas solubility, such as water and water-soluble organic solvents.20–22 Polarity difference changes resulted in gas accumulation which escapes in the form of bubbles.23 To obtain nanosized O2 bubbles, the air (mainly N2 and O2) dissolved in water and water-soluble organic solvents was removed using a vacuum pump. These solvents were replenished with O2 to form O2-rich solutions respectively.

After mixing oxygenated water and four water-soluble alcohols respectively, O2 solubility in these mixtures was monitored using an O2 dissolving meter. The results revealed that O2 solubility decreased and the biggest change was the mixture of n-propanol and water (Fig. 1A–D). The excess O2 escaped from methanol/water, ethanol/water, isopropanol/water, and n-propanol/water was 3.71 mL L−1, 5.46 mL L−1, 4.97 mL L−1 and 7.14 mL L−1, respectively. We further studied the size and morphology of O2 bubbles by taking n-propanol/water as an instance at different time points. As shown in Fig. 1E, the dynamic light scattering (DLS) distribution showed that the sizes of O2 bubbles were uneven, ranging from 0.7 nm to 5369 nm. These small O2 bubbles were fragile and fused with other bubbles to form larger bubbles. Interestingly, these O2 bubbles with an average size of 188.5 nm remained in existence stably for 10 min. Their generation, fusion and escape processes were also confirmed using a microscope (Fig. 1F).

The polymerization rate of DA monomers at the gas–liquid interface is two times faster than that in solution.24 Therefore, we speculated that O2 bubbles are promising as a template for guiding DA polymerization to fabricate HPDA. To prove this hypothesis, after mixing oxygenated water and oxygenated n-propanol, DA monomers were added to the mixing solution. The PDA formation process was monitored using a transmission electron microscope (TEM). As shown in Fig. 2A, DA cyclization and covalent cross-linking to form composite films at the gas–liquid interface occur. The Raman spectra confirmed that HPDA was similar to eumelanin (Fig. S1†). Compared with PDA nanobowls, HPDA would not break with the increasing pressure inside the O2 bubbles.25 However, after removing the O2 bubble template, DA polymerized to form PDA nanospheres (Fig. S2†). We further utilized the O2 bubble template generated by mixing other oxygenated water-soluble organic solvents (methanol, alcohol, and isopropanol) with oxygenated water to prepare HPDA successfully (Fig. S3†), indicating the general applicability of the O2 bubble template for guiding DA polymerization. The shell thickness of HPDA could be easily controlled by adjusting the reaction time (Fig. S4†).

Furthermore, HPDA was designed as a carrier for ZnPc via boric acid ester to fabricate HZNPs. As shown in Fig. 2B and C, HZNPs were observed by using TEM and a scanning electron microscope (SEM), and HZNPs presented regular hollow structures with an average size of 156.1 nm (Fig. 2E). The elemental mapping patterns of HZNPs showed that C, N and O were derived from HPDA and ZnPc while Zn was derived from ZnPc, confirming the successful preparation of HZNPs (Fig. 2D). In addition, HZNPs showed UV-vis absorption in the B-band (200–300 nm) and Q-band (600–800 nm), which was attributed to the
characteristic $\pi-\pi^*$ electron transition of ZnPc. The broadband absorption ability of HPDA resulted in the increasing absorbance of HZNPs (Fig. 2F). As shown in Fig. 2G, ZnPc loading in HZNPs also increased the zeta potential of HZNPs. The inductively coupled plasma spectrometer (ICP) data indicated that the average loading efficiency of ZnPc in HZNPs was 0.2891 mg mg$^{-1}$ (Table S1†). To further study the stability of HZNPs, the DLS distribution of HZNPs was detected in PBS and the cell culture medium. As shown in the DLS results, no obvious HZNP aggregation was detected in PBS or DMEM+10% bovine serum, indicating its good stability in these bio-surroundings (Fig. S5†).

HPDA with abundant catechol could form borate ester bonds with ZnPc, and the pH-responsive borate ester bond was broken under acidic conditions. The vigorous metabolism and profound hypoxia in tumour tissue led to cell glycolysis and superfluous acid substance generation. Then, pH-triggered ZnPc release behaviours of HZNPs were studied under acidic conditions. As shown in Fig. 3A and S6†, the release rate of ZnPc from HZNPs increased obviously under acidic conditions while a slight change was observed under normal conditions, and the ZnPc release rate reached 64.9% when the pH is 5.5, which will recover PDT-mediated antitumor activity (Fig. 3A, S6 and S7†).

Even though HPDA has good stability due to its extensive covalent and noncovalent interactions, HPDA could be degraded in H$_2$O$_2$ aqueous solution because the –OH group in HPDA was oxidized to C=O and –COOH groups (Fig. S8†). To study the encapsulation ability of HZNPs for O$_2$, the Ru(dpp)$_3$Cl$_2$ (H$_2$O$_2$, 200 μM) was used to detect the O$_2$ levels of different samples. As shown in Fig. 3B and S9†, the fluorescence intensity of the HPDA and HZNP treated groups decreased obviously while the ZnPc treated group showed a slight change, indicating that HPDA and HZNPs could increase the O$_2$ level under hypoxic conditions. We further investigated the effect of HPDA-mediated PTT on the stability of HZNPs. After being dispersed in H$_2$O$_2$ aqueous solution for 30 min, HZNPs decomposed slightly at room temperature (Fig. S10†). In contrast, after laser irradiation for 30 min, HZNPs decomposed rapidly, revealing that PTT may promote the O$_2$ release and alleviate tumour hypoxia.

Methylene blue (Methylene blue), a pivotal evaluation indicator for the photosensitizer, is positively related to the O$_2$ level. After light irradiation, the O$_2$ generation ability of different drugs was evaluated using 3,3′-(anthracene-9,10-diyl)dipropionic acid (ADPA). As can be seen from Fig. 3C and S11†, the ADPA absorbance bleaching at 378 nm showed that the order of O$_2$ generation efficiency of different drugs was $k_{ADPA}$ (6.71 × 10$^{-3}$) > $k_{ZnPc}$ (1.67 × 10$^{-3}$) > $k_{HPDA}$ (0.39 × 10$^{-3}$) > $k_{Control}$ (0.34 × 10$^{-3}$), indicating that the high O$_2$ generation efficiency of HZNPs was attributed to ZnPc and the increasing O$_2$ level.

Melanin-like PDA has strong photothermal properties, and the photothermal conversion rate ($\eta$) of HZNPs was measured with a photothermal imager. After laser irradiation for 5 min, the temperature of HPDA and HZNP solutions reached up to 67.1 °C and 67.6 °C respectively, while that of ZnPc was 36.2 °C, revealing that the $\eta$ of HZNPs was mainly derived from HPDA (Fig. 3D and E). To calculate the $\eta$ of HPDA, the absorbance of HPDA was detected to be 0.3073 at 808 nm (Fig. S12†). The temperature of HPDA solution raised to the highest upon laser
irradiation, then, the laser was turned off, and the natural cooling temperature of HPDA solution was recorded at different points of time. The cooling time (t) curve versus the negative natural logarithm of temperature driving force (−\ln \theta) is shown in Fig. 3F. The time constant (\tau) for heat transfer in the system was calculated to be 385.73 and the η was determined to be 38.2%. Three photothermal cycles showed that photothermal conversion capability decreased slightly, indicating that HPDA has good stability (Fig. 3G). Furthermore, the photothermal properties of HZNPs were measured in tumour-bearing mice. After laser irradiation for 9 min, the temperature of the right solid tumour reached up to 45.1 °C, suggesting that HZNPs could kill tumour cells by PTT (Fig. 3H and I).

HZNP alleviating hypoxia behaviour in vitro was detected using Ru(dppe)3Cl2 under hypoxic conditions. Compared with others, the fluorescence intensity of the ZnPc treated group increased obviously upon light irradiation because of PDT-depletion of O2. However, even though PDT consumed O2, the HZNP treated group still showed no fluorescence change, which indicated that O2 released from HZNPs alleviated intracellular hypoxia (Fig. 4A and B). O2 is an important component in PDT since the light initiated 1\text{O}_2 generated by photosensitizers needs O2 motivation. Therefore, we speculated that O2-evolving HZNPs could enhance the anticancer activity of PDT. To verify this speculation, Singlet Oxygen Sensor Green (SOSG) was used to measure the intracellular \text{1O}_2 generation ability of different drugs. Under hypoxic conditions, the HPDA and ZnPc treated groups all showed weak fluorescence while the HZNP treated group showed fluorescence enhancement upon light irradiation (Fig. 4C and D). All these results above indicated that HZNPs released O2 and improved the generation ability of ZnPc in tumour cells.

To further elucidate drugs’ anticancer mechanisms, the intracellular localization of ZnPc and HZNPs was measured using a confocal laser scanning microscope. As shown in Fig. 4E, the colocalization overlap of drugs and probes (MitoTracker Green or Lyso-Tracker Blue) suggested that the action site of HZNPs was the lysosome. Cell Counting Kit-8 (CCK8) assay was used to detect drugs’ dark toxicity and phototoxicity under normoxic and hypoxic conditions. First of all, we tested the IC_{50} value of each formulation. The IC_{50} values of light treatments were remarkably lower than those of non-light treated groups, indicating that light was one crucial factor in damaging tumour cells (Table S2†). Furthermore, all drugs showed no cytotoxicity without light irradiation (Fig. 4F). After different light irradiations, the HPDA treated group and ZnPc treated group showed PTT-induced (808 nm) and PDT-induced (665 nm) 4T1 cell growth inhibition effects, respectively. The HZNPs have combined and more efficient PTT and PDT anticancer effects under normoxic conditions (Fig. 4G). In contrast, the PDT-induced cell death rate by ZnPc decreased obviously under hypoxic conditions, and the anticancer efficiency of HZNPs was superior to that of ZnPc, indicating that HZNPs could alleviate tumour hypoxia (Fig. 4H).

The phototoxicity of different drugs in vivo was studied in tumour-bearing mice by monitoring the tumour volume change during 14 days of treatment. The metabolism distribution of ZnPc and HZNPs in mice was monitored using an IVIS Lumina LT system. Compared with the ZnPc treated group, the stronger fluorescence intensity of the HZNP treated group suggested that HZNPs have a better retention effect in solid tumours (Fig. 5A, B and S13†). In addition, the relative amount of the Zn element in the main organs and tumours of tumour-bearing mice was measured by inductively coupled plasma mass spectrometry (ICP-MS). The relative amount of the Zn element in tumours treated with HZNPs was 8.16 mg g⁻¹, 2.6 times that of the ZnPc treated group (3.15 mg g⁻¹). Compared with the ZnPc treated group, HZNPs accumulated in the kidney decreased, which was beneficial to reduce the side effects of ZnPc (Table S3†). Many literature studies reported that nanomedicine accumulated in tumour and inflamed tissue by the enhanced EPR effect. Recent scientific studies indicated the passive accumulation of nanomedicines at the tumour site is not as important as the
HZNPs treated with combination light (808 nm + 665 nm) had the best anticancer efficiency, which was consistent with the results of H&E staining. Furthermore, the hypoxia-inducible factor (HIF)-1α staining assay was used to evaluate the hypoxic condition in the tumours of different groups. The tumour immunofluorescence of ZnPc treated with 665 nm light was stronger than that of control, implying that the O2-depletion of PDT resulted in HIF-1α overexpression. Even if the PDT process consumed O2 in tumours, the immunofluorescence intensity was still weak in the HZNP treated group, indicating that the HIF-1α expression was inhibited by the O2-evolving HZNPs (Fig. 5J).

**Conclusions**

In conclusion, a facile and nanosized O2 bubble template was reported by mixing oxygenated water and water-soluble organic solvents. O2-evolving HPDA was prepared based on the nanosized O2 bubble template, which would inspire the gas bubble template for guiding materials synthesis. The in vitro and in vivo results revealed that HZNPs could release O2 and alleviate hypoxia to enhance the PDT efficiency. Under a combination of light (808 nm + 665 nm) irradiation, HZNPs achieved synergetic and satisfactory anticancer efficiency, and the tumour growth rate was suppressed up to 73.91% when compared to that of control mice. Our study provides a novel strategy for O2 delivery by nanomaterials in cancer treatment.

**Conflicts of interest**

There are no conflicts to declare.

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