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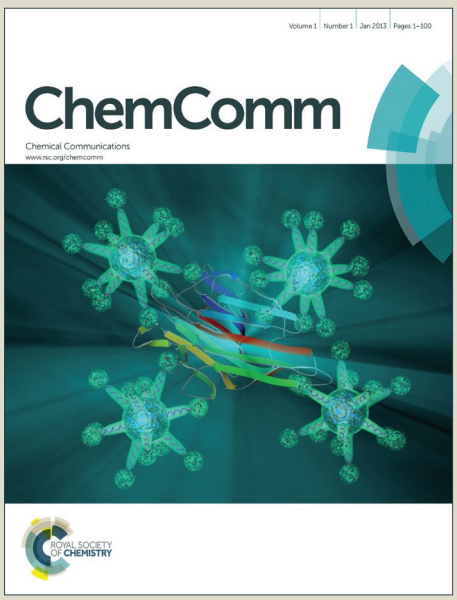

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A H^+-Triggered Bubble-Generating Nanosystem for Killing Cancer Cells

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We constructed a H^+-triggered bubble-generating nanosystem (BGNS), which generated CO_2 bubbles in the acidic environment of lysosomes after internalized by cancer cells.The quickly generated bubbles caused enhanced lysosome membrane permeabilization. As expected, H^+-triggered BGNS possessed remarkable cytotoxicity against MCF-7 breast cancer cells, and successfully overcame the multidrug resistance of MCF-7/ADR cells.

Chemotherapy is one of the most common treatments for cancer; however, multidrug resistance (MDR) remains a significant obstacle for the effective treatment of this disease.[1-3] To address this formidable challenge, diverse cancer therapies, such as photodynamic therapy,[4] nucleic-targeted[5] or nanocarrier-based drug delivery systems,[6] bacteria[7] or viral-based[8] therapy, and short interfering RNAs for cancer gene therapy,[9] have been developed to efficiently overcome the MDR of cancer cells. Lysosomes have been defined as "suicide bags."[10] As lysosome membrane permeabilization (LMP) occurs, the release of certain cathepsins from the lysosome into the cytoplasm is thought to trigger cell death by apoptosis and apoptosis-like pathways.[11,12]So, these organelles have gained much attention as potential therapeutic targets in cancer.[13-15]In this work, we constructed novel H^+-triggered bubble-generating nanosystem (BGNS), which could enhance LMP by generating CO_2 bubbles. The certain cathepsins release from the lysosome into the cytoplasm, triggering cell death by apoptosis and apoptosis-like pathways due to upregulation of active caspase-3. The direct lysosome-targeted BGNS successfully bypassed the drug efflux pump, leading to enhanced apoptosis of MCF-7/ADR cells and the much efficient overcoming of MDR.

Incidentally, we found that doxorubicin (DOX), a widely used antitumor drug, reacts with $NaHCO_3$ to produce a red precipitate (Fig.S1†). In order to eliminate the interference of hydroxyl, we added DOX to sodium hydroxide solution (pH 8.5), which has the same pH as 0.2 $molL^{-1}$ $NaHCO_3$, and found no precipitate (Fig. S1†). This demonstrates that the red precipitate was bicarbonate, and not hydroxide precipitate. In addition, the red precipitate could be dissolved in an acidic phosphate buffer solution (PBS, pH 5.0) and generated bubbles. Based on these findings, we developed pH-responsive BGNS that could enhance LMP by generating CO_2 bubbles.

Among the diverse nanomaterials used in therapeutic systems, hollow mesoporous silica nanoparticles (HMSNs) have substantial significance in nanobiomedical research because of their large surface area, high pore volume, tunable pore sizes, and excellent biocompatibility.[16–19] We chose HMSNs as a platform to establish BGNS. Monodispersed HMSNs can be readily synthesized by an improved selective etching process.[20] Transmission electron microscopy (TEM) showed that the HMSNs possessed a highly uniform and monodispersed spherical morphology with a distinctive hollow nanostructure (Fig.S2a†) and average diameter and wall thickness of 308 nm and 48 nm, respectively. Nitrogen adsorption desorption isotherm measurements showed that they had a specific surface area of 1.04×10^{-2} m^2g^{-1} and well defined pore sizes of 10.2 nm (Fig.S2b†).

To establish BGNS, first we incubated DOX with HMSNs and obtained DOX-loaded HMSNs (DMSNs), after which we treated the DMSNs with $NaHCO_3$. The loading efficiency of DOX in DMSNs and BGNS was 5.3% and 16.8%, respectively. The electrostatic interaction between the positively charged DOX molecules and negatively charged silica surface is the main force for drug loading.[21] After treating the DMSNs with $NaHCO_3$, the loaded DOX molecules formed DOX-HCO_3 precipitate and leave from interior silica surface to the hollow cavity. Thus, the silica surface of BGNS was able to load more DOX.

The release rate of DOX from BGNS was lower than that from DMSNs in PBS at pH 7.4, which simulated normal physiological conditions (Fig. 1a). Conversely, the release of DOX from BGNS was faster than that from DMSNs in PBS at pH 5.0, which simulated lysosomal conditions. These results suggest that upon internalization by cells, BGNS can release DOX faster than DMSNs in acidic

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† Electronic Supplementary Information (ESI) available: Detailed experimental procedures and materials; Fig. S1 – S3

intracellular compartments, whereas BGNS showed less drug leakage than DMSNs in normal physiological conditions.

TEM showed a corresponding result with the drug release profile. After incubating DMSNs with PBS at pH 5.0 for 24 h, fewer nanoparticles with a hollow structure could be seen (Fig.S3a†). However, after incubating BGNS with PBS at pH 5.0 for 24 h, more particles with a hollow structure could be observed (Fig.S3b†). Treating both DMSNs and BGNS with PBS at pH 7.4 for 24 h led to the observation of almost no hollow structure. For the DMSNs, DOX was adsorbed onto the silica surface mainly via electrostatic attraction, and the pH-controlled release was attributed to a decrease in attraction because DOX can exchange with protons under acidic conditions.[21] For BGNS, DOX was transformed into DOX-HCO_3, which is insoluble in water and neutral solution. However, BGNS strongly reacted with H^+ and generated a lot of CO_2 bubbles in PBS at pH 5.0 (Fig.S3b†). As reported,[22,23] the generated bubbles accelerated the release of DOX.

To further determine if BGNS could enhance the anticancer efficacy against MCF-7 cells, the cytotoxicity of free DOX, DMSNs, and BGNS was measured by CCK-8 assay at different DOX concentrations. Free DOX, DMSNs, and BGNS all showed dose-dependent toxicity, with BGNS having the highest toxicity (Fig. 1b–c). The half-maximal inhibitory concentration (IC_{50}) of free DOX, DMSNs, and BGNS against MCF-7 cells after 24 h of treatment was 5.51, 4.50, and 3.96 μgmL^{-1}, respectively (Fig. 1b), and after 48 h of treatment was 3.61, 3.35, 2.66 μgmL^{-1}, respectively (Fig. 1c). As expected, the BGNS substantially enhanced cytotoxicity towards MCF-7 cells.

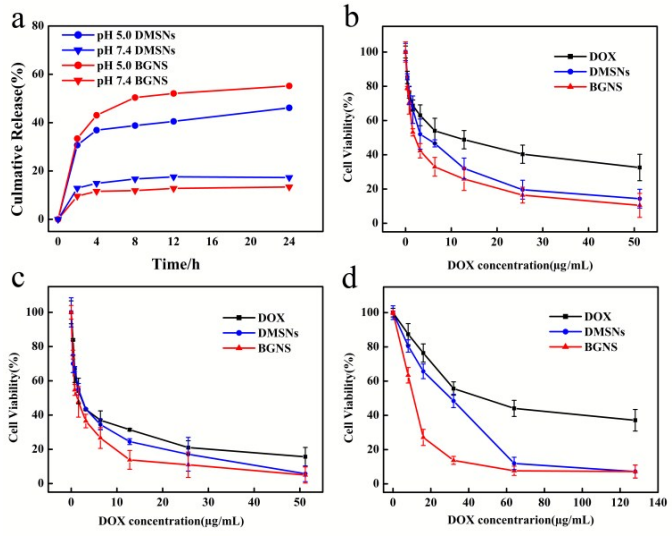

Fig.1 Drug release and anticancer effects against MCF-7 and MCF-7/ADR cells. a, The release profiles of DOX from DMSNs, BGNS in different PBS with pH 7.4 and pH 5.0 at 37 °C. b, c, Cell viability of MCF-7 cells after incubating with free DOX, DMSNs, or BGNS at different concentrations for 24 h (b) and 48 h (c). d, Cell viability of MCF-7/ADR cells after incubating with free DOX, DMSNs, or BGNS at different concentrations for 48 h.

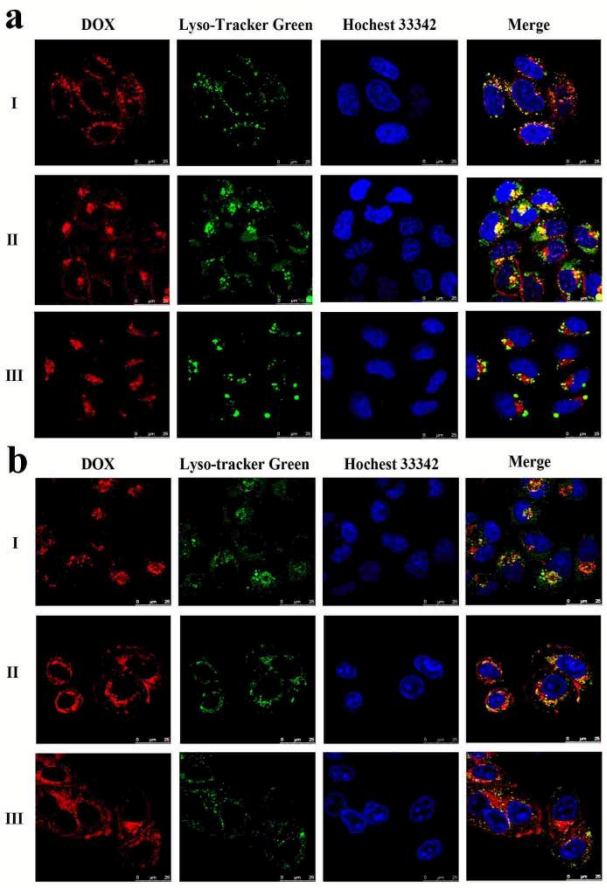

Fig.2 Cellular colocalization images. a, b, CLSM images of the intracellular accumulation of DOX in MCF-7 cells after incubating with Free DOX (I), DMSNs (II), or BGNS (III) for 4 h (a), 24 h (b).

To investigate the intranuclear distribution of DOX in cells, we used confocal laser scanning microscopy (CLSM) to visualize its intracellular localization in MCF-7 cells incubated with free DOX, DMSNs, or BGNS. For free DOX and DMSNs, red fluorescence from DOX could be observed in the lysosomes and nuclei within 4 h (Fig. 2a). However, with BGNS, the red fluorescence mostly accumulated in the lysosomes, indicating that little DOX got into the MCF-7 cell nuclei. The distribution of DOX did not change with a longer incubation time (Fig. 2b). Interestingly, the DOX of BGNS always accumulated in the lysosomes rather than in the cell nuclei. It is important that DOX is transported into the cell nuclei to exert its cytotoxicity, because its mechanism of action is to interact with DNA by intercalation and inhibit macromolecular biosynthesis.[24-26]

Even though the DOX of BGNS accumulated in the lysosomes rather than in the cell nuclei, BGNS killed MCF-7 cells more effectively than DMSNs, which can transport DOX to the nuclei of cancer cells. An interesting question is how BGNS were able to effectively kill cancer cells without the aid of DOX. BGNS can be endocytosed by MCF-7 cells and trapped in lysosomes (Fig. 2). Due to the acidic conditions inside lysosomes, DOX-HCO_3 inside BGNS strongly reacted with H^+ and generated lots of CO_2 bubbles (Fig. S3b†).

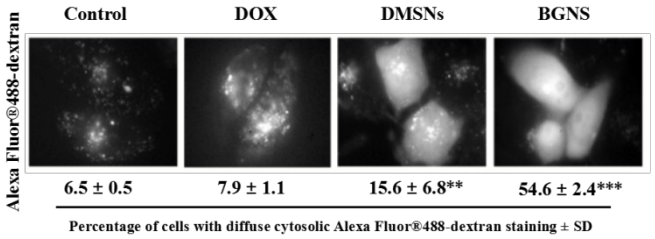

Fig.3 Visualization of LMP. **p < 0.01, and ***p < 0.001, when comparing drug treated cell with untreated cells (control); and p < 0.001, when comparing BGNS treated cells to DMSNs treated cells.

To investigate the effects of bubbles on the lysosomes, changes in LMP were visualized by fluorescence imaging according to a previously reported method.[14] With increasing LMP, fluorescent dextran progressed from a punctate lysosomal to diffuse cytosolic staining. Free DOX had almost no effect on changes in LMP, and DMSNs slightly increased LMP due to the "proton sponge" effect (Fig. 3).[27,28] BGNS dramatically increased LMP (Fig. 3), which could be attributed to BGNS acted as explosives as they generated numerous CO_2 bubbles inside the lysosomes of MCF-7 cells.

Increased LMP may cause the release of certain cathepsins from the lysosome into the cytoplasm, and can trigger death by apoptosis and apoptosis-like pathways.[11] Caspase-3 is a key executor of apoptosis, the upregulation of active caspase-3 is an evidence of apoptosis.[29,30] After incubation with free DOX, DMSNs, and BGNS, caspase-3 activity in MCF-7 cells was measured. Free DOX and DMSNs induced a slight increase, whereas BGNS induced the highest increase in caspase-3 activity (Fig. 4). The higher the value of caspase-3, the more apoptosis of cancer cell. These results are in accordance with the cytotoxicity results showing that BGNS had the highest anticancer activity (Fig. 1b–c), and implicated that apoptosis was the mechanism underlying cell death.

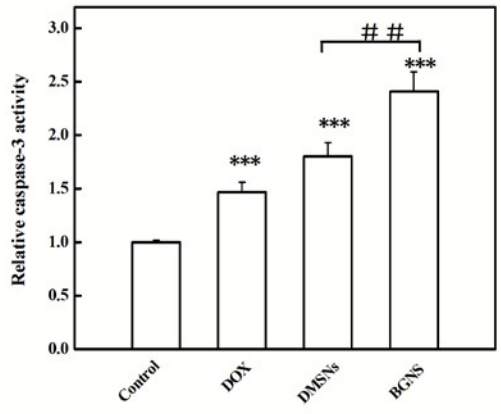

Fig.4 Caspase-3 activities. Cells were incubated with medium (control), free DOX, DMSNs, and BGNS for 48 h. The concentration of DOX was 1 μgmL^{-1}. ***p < 0.001, when comparing drug treated cell with untreated cells (control); ## p < 0.01, when comparing BGNS treated cells to DMSNs treated cells.

Sung's group constructed CO_2 bubble-generating drug delivery systems based on liposome and poly(lactic-co-glycolic acid) (PLGA).[22,31-34] The PLGA-based system was pH-responsive and can prompt release of DOX once internalized in lysosome of cancer cells.[22,31] The liposomal system was thermoresponsive and can prompt release of drug[32,33] or lead to cell necrosis[34] by the generating bubbles when heated locally. Our HMSN-based BGNS was pH-responsible and can kill cancer cells directly by bubbles without the aid of heat.

Some cancer cells acquire the ability to efflux drugs by increasing the expression of MDR proteins such as P-glycoproteins (P-gps) of the ATP-binding cassette transporter family.[2]BGNS can kill cancer cells without the aid of DOX, and as such, are able to overcome the MDR of cancer cells. MCF-7/ADR cells were used to test the toxicity of BGNS. As expected, BGNS substantially enhanced cytotoxicity towards MCF-7/ADR cells (Fig. 1d). The viability of MCF-7/ADR cells did not considerably decrease at low concentrations of free DOX. Even after a 48 h incubation with a relatively high DOX concentration of 16 μgmL^{-1}, there was an approximately 70% survival rate of MCF-7/ADR cells. The DMSNs showed limited enhancement of anticancer efficiency compared to free DOX, which might be attributed to nanoparticle-mediated DOX delivery mechanism to bypass P-gp-mediated efflux.[19] However, only 28% of MCF-7/ADR cells survived when 16 μgmL^{-1} BGNS was incubated with MCF-7/ADR cells for 48 h. Significantly decreased IC_{50} values of BGNS are shown in Fig. 1d, IC_{50} of free DOX, DMSNs, BGNS against MCF-7/ADR cells after 48 h treatment were 45.6, 22.5, and 10.6 μgmL^{-1}, respectively. These results confirm that BGNS can effectively kill MDR cancer cells. In summary, we developed and characterized BGNS, which had remarkable anticancer effects against MCF-7 and MCF-7/ADR cells. BGNS could enhance LMP by releasing CO_2 bubbles in the intracellular lysosome, whereas they are stable elsewhere in the body. BGNS showed the greater anticancer effects than free DOX and DMSNs, and may be a potential candidate for overcoming MDR.

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