Macromolecular Research Article

DOI 10.1007/s13233-017-5078-9 www.springer.com/13233 pISSN 1598-5032 eISSN 2092-7673

Novel Chemi-Dynamic Nanoparticles as a Light-Free Photodynamic Therapeutic System for Cancer Treatment

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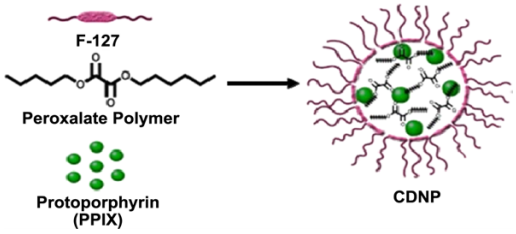
Received January 9, 2017 / Revised March 6, 2017 / Accepted March 7, 2017

Abstract: Photodynamic therapy (PDT) is a treatment modality, in which photosensitizers are activated by light of appropriate wavelength to convert molecular oxygen from triplet state ($^3O_2$) to singlet ($^1O_2$) that kills nearby cancer cells. However, the clinical application of PDT is limited to treat tumors on or just below the skin or the lining of internal organs because the light needed cannot pass through more than 1 cm of tissues. Here, we report chemi-dynamic nanoparticles (CDNP) as a light-free photodynamic therapeutic system based on peroxalate chemiluminescence. Photosensitizers are excited by the energy generated from hydrogen peroxide-mediated degradation of peroxalate to generate singlet oxygen to kill cancer cells. CDNP kill cancer cells without external light and hold potential as new anticancer drugs.



Keywords: photodynamic therapy, peroxalate chemiluminescence, cancer, micelles, chemi-dynamic therapy.

1. Introduction

Cancer is a chronic pathological condition which engenders one in four deaths in the United States[1] and global expense in cancer management has been rising annually.[2] Development of effective and efficient treatment strategies to fight cancer is an aspiration of researcher communities around the world. Photodynamic therapy (PDT) is a treatment modality that employs an extrinsic photosensitive compound (photosensitizer) that could mediate a photochemical reaction involving the conversion of molecular oxygen from triplet state ($^3O_2$) to singlet ($^1O_2$) in the presence of light of appropriate wavelength.[3] PDT has been known for a century since Oscar Raab demonstrated the cytotoxic effect of fluorescent compounds in *Paramecium caudatum.* PDT was initially developed to treat a microbial infection like lupus vulgaris, from where its application gradually extended to treat early stage cancer.[4] Although there are a couple of advantages with PDT, poor penetration of light into deep tissues limits the applications of PDT for various types of tumors.[5] Photosensitizers currently utilized for PDT require external light such as laser or non-laser sources.[6,7] The majority of the photoemission by a laser light delivery systems is partially absorbed and attenuated by surrounding tissues. Therefore, light delivery to the tumor interstitium is imperceptible and remains a major challenge.[8] In order to expand clinical applications of PDT, it is critical to developing new strategies to activate photosensitizers inside cells without external light source. In this context, intracellular generation of light in tumor sites would be a great alternative to light delivery.

Chemiluminescence is the chemical production of light as a result of chemical reactions. One example of chemiluminescence reactions is the oxidation of luminol, which is the best known and most efficient light emitting reaction.[9] Emission of light catalyzed by enzymes in a biological system is bioluminescence, one of sub-types of chemiluminescence.[10] The chemical light emission in these reactions is achieved by nonradioactive energy transfer from chemiluminescent and bioluminescent donors to suitable acceptor molecules.[11] To date, several researchers have developed "self-illuminating" PDT systems, in which photosensitizers are excited by chemiluminescent resonance energy transfer (CRET) or bioluminescent resonance energy transfer (BRET), not by external light sources.[11] Luminol in basic solutions could be oxidized by hydrogen peroxide (H_2O_2) in the presence of ferrous sulfate as a catalyst to activate photosensitizers.[12] Luciferin could also excite photosensitizers in cells transfected with luciferase gene. Despite their great promise as excitation sources for PDT in the proof-of-concept studies, luminol-based chemiluminescence and luciferin-based bioluminescence have several limitations including the toxicity of luminol, need of luciferase expressing cells and insufficient photon generation.[11-13] We therefore sought to investigate the potential of peroxalate chemiluminescence (POCL) as an energy source to excite photosensitizers.

POCL is a sub-type of chemiluminescence, in which peroxalate ester compounds are oxidized by H_2O_2 to form unstable dioxetanedione of high energy, which is further decomposed

Acknowledgments: This work was supported by a grant of Korean Health Technology R&D Project (HI15C1619), Ministry of Health & Welfare and Basic Science Research Program (2016R1A2B4008489) through National Research Foundation funded by the Ministry of Education, Republic of Korea.

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into carbon dioxide and energy.[14] H_2O_2 is the primary oxidant required to trigger POCL. Interestingly, cancer cells have a higher level of H_2O_2 than normal cells to enhance cell proliferation and maintain the cancer phenotype.[15] However, various anticancer drugs are also known to induce the massive generation of reactive oxygen species (ROS) including H_2O_2 in cancer cells. The overproduction of ROS pushes the oxidative level beyond the cancer cell survival threshold, eventually leading to apoptotic cell death.[16,17] In addition, it has been reported that energy from POCL reaction is able to excite various fluorescent dyes of broad ranges of emission wavelengths.[18] On the basis of the H_2O_2-triggered energy generating features and excellent H_2O_2-specificity of POCL, we hypothesized that energy generated from H_2O_2-triggered oxidation of peroxalate ester could excite photosensitizers to generate highly toxic $^1O_2$ for cancer cell death. Herein, we report a novel light-free photodynamic anticancer therapeutic system, in which peroxalate-based polymer is exploited as a source of energy. To our best knowledge, no attempts have been yet made to utilize the principle of POCL as an energy source for PDT in cancer treatment.

In our laboratory, we previously developed hydroxybenzyl alcohol-incorporating copolyoxalate (HPOX) as an H_2O_2-responsive antioxidant and anti-inflammatory polymer, in which peroxalate ester linkages are covalently incorporated into its backbone.[19-21] HPOX nanoparticles could effectively react with H_2O_2 and exert potent antioxidant and anti-inflammatory activities. Chemiluminescent polymeric micelles composed of HPOX and fluorescent dyes could image H_2O_2 generated during inflammatory responses by performing POCL with excellent sensitivity and specificity.[22] In this work, we developed CDNP using HPOX as a framework of peroxalate ester and protoporphyrin (PPIX) as a photosensitizer and evaluated its therapeutic efficacy using a cell culture model.

2. Experimental

2.1. Synthesis of HPOX

In 20 mL of anhydrous tetrahydrofuran (THF), 1,4-cyclohexanedimethanol (21.96 mmol) and 4-hydroxybenzyl alcohol (5.49 mmol) were added and triethylamine (60 mmol) was added dropwise. Oxalyl chloride (27.45 mmol) dissolved in THF was added dropwise to the mixture and the polymerization reaction was carried out at low temperature (4 °C) for 6 h under a nitrogen purge. Sodium chloride solution was added to terminate the reaction and the solvent was removed under a low pressure in a rotary evaporator. The polymer was precipitated in cold hexane and the solvent was removed using a rotary evaporator. Pure polymer was obtained by vacuum drying. The chemical structure of the polymer was determined using proton NMR 400 MHz (JNM-EX400, JEOL, Japan).

2.2. Preparation and characterization of CDNP

In 1 mL of THF, Pluronic F-127, HPOX and PPIX were dissolved at a weight ratio of 10:1:0.5. The mixture was added in 20 mL distilled water and the solvent was removed using a rotary evaporator to form micelles through self-assembly. The size and polydispersity of the micelles were measured using dynamic light scattering (DLS, ELS-6000, Photal Otsuka Electronics, Japan).

2.3. Stability of CDNP

CDNP-micelles were freshly prepared then incubated with 100% FBS and PBS pH 7.4 at 37 °C for 24 h. Samples were analysed at pre determined time 0 h, 3 h, 6 h, 12 h, and 24 h interwals using DLS. After analysing DLS, few microliters of samples were transfered to a TEM grid, followed by negative staining with a tungstic acid solution. Samples were dried and were analysed with a transmission electron microscope (TEM, JEOL-2010, Japan).

2.4. Determination of singlet oxygen generation by CDNP

CDNP reactivity to hydrogen peroxide was observed by using DMA 9,10-dimethylanthracene as a singlet oxygen probe. DMA was added in distilled water in the absence or presence (100 μM) of hydrogen peroxide. CDNP was added to the mixture and the fluorescence emission spectrum of DMA was recorded using a photoluminometer (Jasco, Japan).

2.5. Cell culture

Cell culture study was performed using human colon cancer cell line (SW620) obtained from Korea Cell Line Bank. Culture medium RPMI 1640 (Gibco, BRL, USA) containing 10% fetal calf serum (FBS, Gibco, BRL, USA) and 1% antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) was used. Cells were incubated with 5% CO_2 at 37 °C and the culture medium was replaced every three days.

2.6. Cytotoxicity and morphological evaluation

To evaluate the cytotoxicity of CDNP, SW620 cells were seeded in a 96 well plate at a density of 3×10^5 cell per well and cultured in 5% of CO_2 at 37 °C for 24 h. Cells were treated with various formulations for 24 h. Media were removed and 100 μL of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (diluted in a culture medium to a final concentration of 0.5 mg/mL) was added and incubated for another 4 h. MTT reagent was replaced with 100 μL of DMSO (Dimethylsulfoxide, Sigma-Aldrich) and fluorescence emission was monitored using a microplate reader (Biotek Instruments, Winooski, BT) at wavelength 570 nm. Cytotoxicity was expressed as the percentage of cell viability compared to untreated control cells. Each data point is represented as mean ± standard deviation (SD) of three independent experiments (n=4).

For the morphological assessment of CDNP, SW620 cells were seeded in a 6 well plate a density of 1×10^6 cell per well and cultured in 5% of CO_2 at 37 °C for 24 h. After 24 h of treatment with various formulations, morphology was observed using an optical microscope (Eclipse TE 2000-U, Nikon, Japan).

2.7. Apoptotic assessment by flow cytometry

SW620 cells were seeded at a density of 3×10^5/well and incu-

bated for 24 h. Cells were treated with various formulations for 24 h. After the incubation end point, the cells were washed twice with PBS, 1×10^5 cells/mL were suspended in 1× binding buffer solution. To a fresh 5 mL FACS tube 100 μL of cell suspension was transferred, propidium iodide and annexin V-FITC (5 μL) was added. The mixture was incubated at room temperature for 15 min and then 400 μL of 1× binding buffer was added. Each sample concentration was maintained to 1.0×10^4 cells, stained cells were analyzed via FL-1 and FL-2 using flow cytometry (FACS caliber, Becton Dickinson, San Jose, CA).

2.8. Confocal laser scanning microscopy

SW620 cells (5×10^5) were cultured in a glass bottom dish (MatTek Corp., Ashland, MA) cells were pre-treated with cinnamaldehyde for 12 h followed by CDNP was treated at various time points 1 h, 6 h, and 24 h, respectively. With fresh medium, cells were washed twice and analyzed using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss, Inc. Germany).

2.9. Immunoblot analysis

Immunoblot analysis was performed to measure the level of protein expression. SW620 cells (1.5×10^6 cells/well) were seeded a 6 well plate and incubated for 24 h. Cells were treated with various formulations for 24 h. Using a lysis buffer soluble proteins were extracted from the cells. Subsequently by using 20 μg of cell lysate polyacrylamide gel electrophoresis (12% SDS-PAGE gel) was performed, resultant proteins were transferred to a polyvinylidene fluoride membrane (BIO-RAD, Sequi-Blot, USA). Transferred membrane was incubated with immunoblot analysis with antibodies to caspase-3 (Santa Cruz Biotechnology, Dallas, TX) Afterwards, the membranes were washed and then reblotted with a β-actin antibody. HRP-conjugated goat anti-mouse (Millipore, Billerica, MA) was used as a secondary antibody, immunoblot signals were developed with chemiluminescent reagent Super Signal Ultra (Pierce, Rockford, IL).

2.10. Statistical analysis

Data were expressed as mean ± standard deviation (s.d.). Comparison between groups was conducted with one-way ANOVA using GraphPad Prism 5.0 (San Diego, CA). Probability (p) values of <0.05 were considered statistically significant.

3. Results and discussion

3.1. Design and characterization of CDNP

The strategy to construct CDNP as a novel anticancer therapeutic system is illustrated in Figure 1. Since HPOX is the energy producing source, it is critical to hold HPOX and PPIX in a close proximity to maximize the efficiency of energy transfer for excitation of PPIX. Therefore, biocompatible amphiphilic copolymer, Pluronic F-127 was used as a scaffold for CDNP. Amphiphilic Pluronic F-127 could self-assemble to form thermodynamically stable micelles, in which HPOX and PPIX were sequestered nearby in their hydrophobic core.[23,24] H_2O_2 would impregnate into the CDNP and thereby reacts instantaneously with HPOX to generate 1,2-dioxethanedione as an intermediate of high energy. Highly unstable 1,2-dioxethanedione rapidly decomposes and releases energy which could activate PPIX to generate $^1O_2$ that ultimately kills the cancer cells: we termed this POCL mimic event as chemi-dynamic therapy (CDT). Dynamic light scattering and transmission electron microscopy were employed to study the structure and mean hydrodynamic diameter of CDNP (Figure 2(a)-(d)). CDNP prepared in aqueous solutions was spherical and showed a mean diameter of ~200 nm. In the physio-

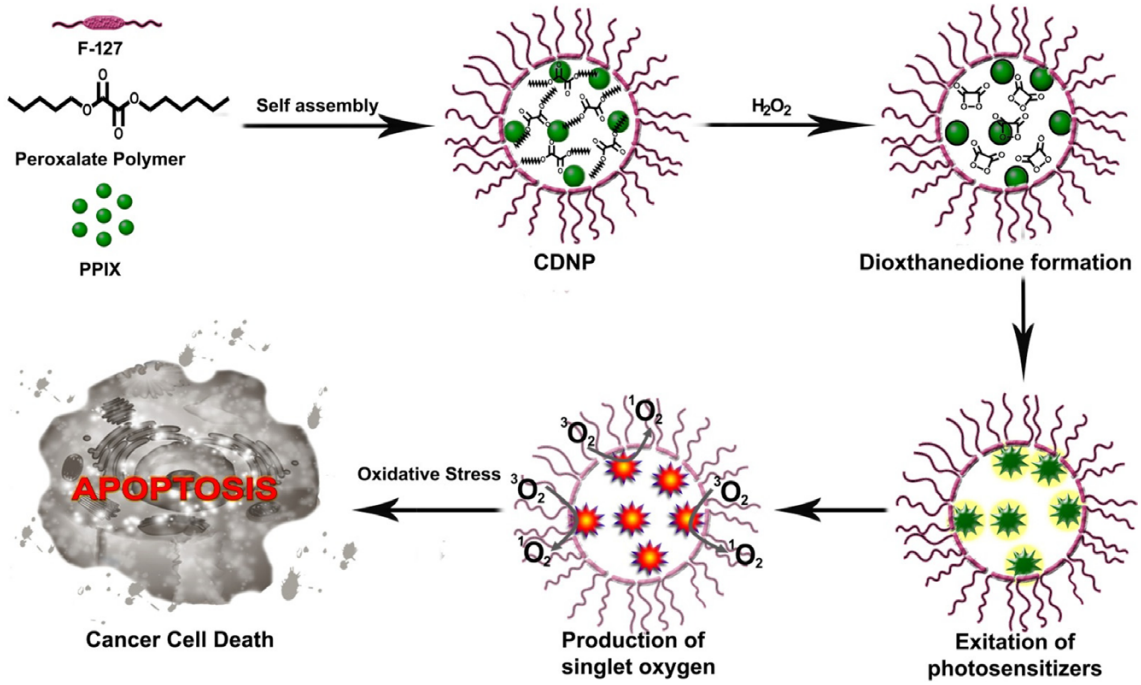

Figure 1. A schematic diagram of light-free PDT using CDNP, in which photosensitizers are excited by energy released from H_2O_2-triggered degradation of peroxalate esters to generate toxic $^1O_2$, leading to apoptotic cancer cell death.

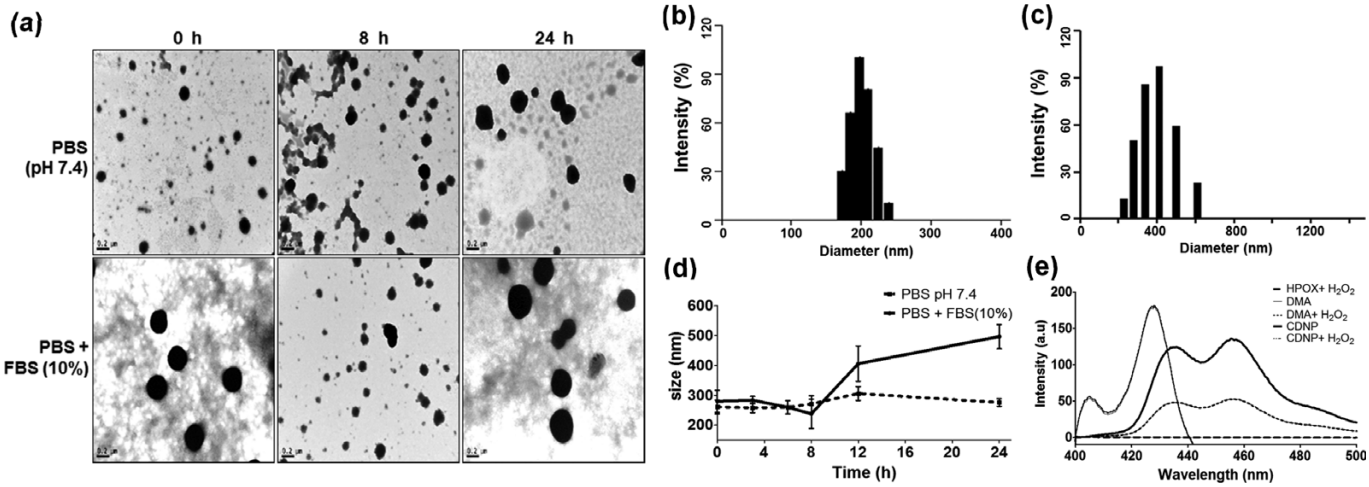

Figure 2. Characterization of CDNP. (a) A representative TEM images of CDNP suspended in phosphate buffer with or without serum protein (10%). (b) A representative dynamic light scattering of CDNP under aqueous conditions without serum protein. (c) A representative dynamic light scattering of CDNP after 24 h of incubation with serum protein (10wt%). (d) Changes in the hydrodynamic diameter of CDNP suspended in phosphate buffer as a function of time. Values are mean±s.d. (n=4). (e) The effects of CDNP on the fluorescence emission of DMA in the presence and absence of H_2O_2.

logical environment containing proteins, the diameter of CDNP was increased due to non-specific protein adsorption. Figure 2(c) shows the hydrodynamic diameter of CDNP after 24 h of incubation with serum protein (10%). Despite the increment of hydrodynamic diameter with time, CDNP maintained the structural integrity for 24 h (Figure 2(d)), demonstrating their physical stability under physiological conditions. The ability of CDNP to generate $^1O_2$ was examined using 9,10-dimethylantracene (DMA) as a probe for $^1O_2$. Fluorescent DMA is known to react with $^1O_2$ to generate non-fluorescent 9,10-endoperoxide with a very high rate constant.[25] We investigated the fluorescent emission of DMA which was added to the suspension of CDNP in the presence or absence of H_2O_2. As shown in Figure 2(e), DMA exhibited a strong emission peak at ~430 nm. The addition of H_2O_2 induced no change in the fluorescence intensity of DMA, indicating that the fluorescence of DMA is not affected by H_2O_2. However, when DMA was added to the suspension of CDNP, the change in the fluorescence was observed. The mixture of DMA and CDNP showed a strong emission at 460 nm, a red shift due to the interaction of DMA and CDNP. The addition of H_2O_2 (100 μM) to the mixture of DMA and CDNP caused significant (~60%) reduction in the fluorescence emission intensity. The results indicate that CDNP generates $^1O_2$ by performing H_2O_2-triggered POCL.[26] The mechanism for excitation of photosensitizers within CDNP could be explained by chemiluminescent energy transfer.[11]

3.2. Cellular uptake and cytotoxicity of CDNP

In order to confirm the cellular uptake of CDNP, CDNP-treated SW620 cells (human colorectal adenocarcinoma) were observed using a confocal laser scanning microscope. Red fluorescence was observed in cells from 1 h after the addition of CDNP and remarkably strong red fluorescence was observed at 6 h (Figure 3(a)), demonstrating that CDNP would be taken up by cells. To investigate whether CDNP could induce apoptotic cell death by photo-induced toxicity, we studied the effect of CDNP on the viability of SW620 cells. Figure 3(b) illustrates the cell viability after the addition on CDNP. HPOX showed no cytotoxicity. CDNP did not exert notable cytotoxicity probably because the level of H_2O_2 is not sufficient to oxidize the peroxalate esters to generate highly toxic $^1O_2$. We, therefore, added exogenous H_2O_2-generating cinnamaldehyde (CA) to cells, which is known to exert anticancer activity by generating ROS mainly H_2O_2.[27] Moderate cytotoxicity was observed with cells treated with CA alone. the CA-induced toxicity is in agreement with our previous studies.[19,28] However, interestingly, co-treatment with CA and CDNP exhibited significant toxicity, in a concentration-dependent manner. More than half of cells were killed by the co-treatment of CA with 100 μg of CDNP. The results indicate that oxidation of peroxalate esters in HPOX is triggered by the elevated level of H_2O_2 to yield sufficient energy which could activate PPIX to generate highly cytotoxic $^1O_2$.

3.3. The effects of CDNP on cell morphology

Morphological assessment using an optical microscope was carried out to further examine the effects of $^1O_2$ on SW620 cells (Figure 4). Cells treated with HPOX, PPIX or CA exhibited confluency that was comparable to the untreated group, with no significant detrimental changes in morphology. CDNP treatment also induced marginal changes in the morphology of cells. However, the group treated with both CA and CDNP showed a greater number of the cells detached from the substrate due probably to the loss of plasma membrane integrity. In addition, a great extent of cell shrinkage was observed with a majority of the detached cells, a hallmark of apoptosis.[29,30] Compared to the other treatment groups, cells treated with both CA and CDNP exhibited remarkable changes in the cell morphology, indicating significant anticancer activity of the CDNP in combination with H_2O_2-generating CA.

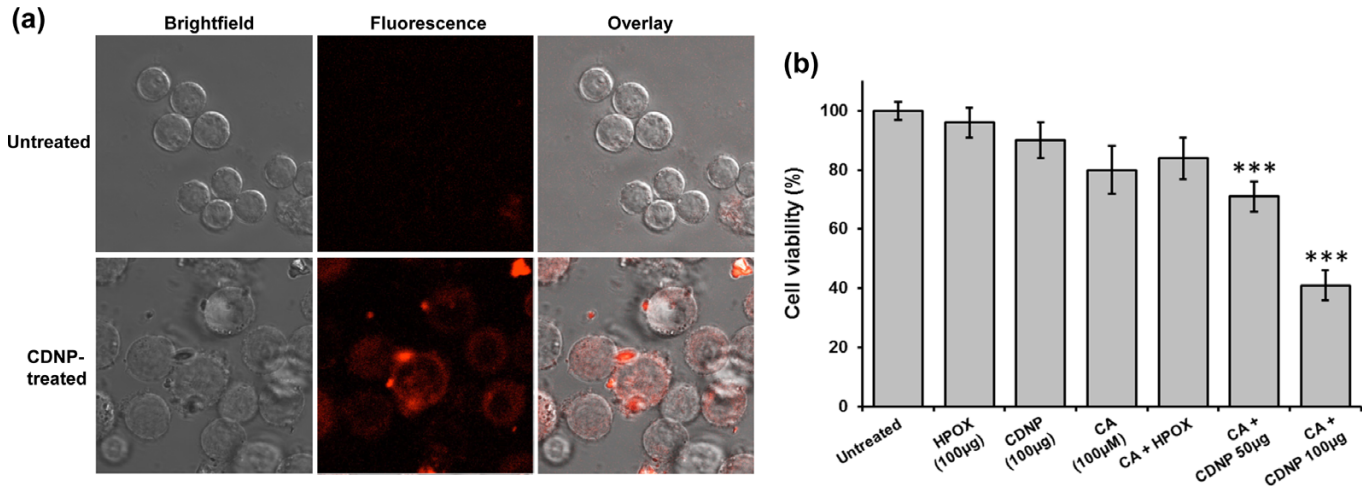

Figure 3. Cellular uptake and cytotoxicity of CDNP. (a) Representative confocal laser scanning micrographs of SW620 cells treated with CDNP for 6 h. (b) Cell viability assay of SW620 cells. Cells were treated with various formulations for 12 h before analysis. *p<0.001 relative to the untreated group. Values are mean±s.d. (n=4).

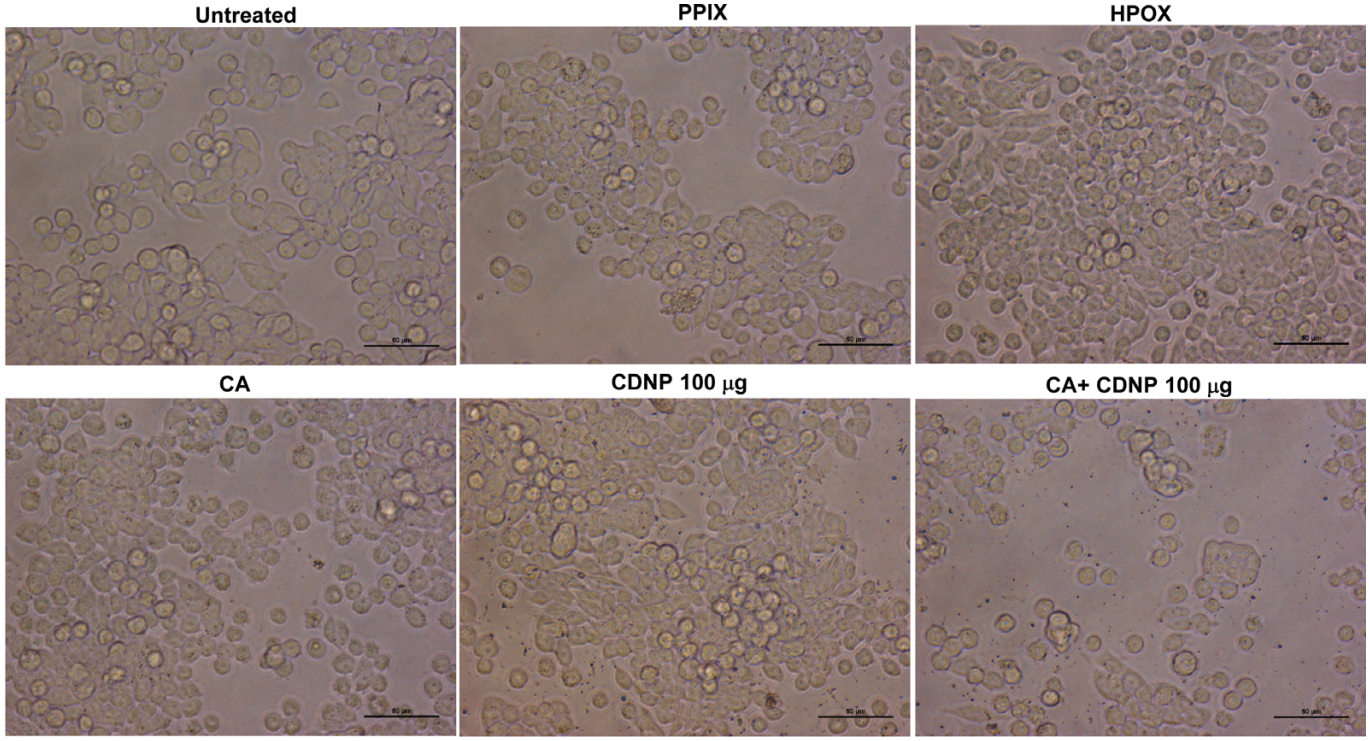

Figure 4. Morphological assessment of SW620 cells. Cells were treated with a respective formulation for 24 h and then examined under a confocal laser scanning microscope. Data shown are representative of three independent experiments.

3.4. CDNP-induced apoptotic cell death

In order to further investigate the mechanism of CDNP-induced cell death, flow cytometric analysis was performed using Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI) (Figure 5(a)). HPOX alone and PPIX alone induced minimal apoptotic damages to cells, as shown by slightly increased populations in the upper right quadrant corresponding to late apoptotic cells. Treatment of CA exhibited a moderate level of pro-apoptotic activity. However, the addition of CDNP in CA-pretreated cells induced significant apoptosis in a dose-dependent manner. For example, CDNP of 100 μg/mL induced apoptosis for half of the cells. These observations demonstrate that even in the absence of an external light source, CDNP could excite photosensitizers to induce apoptotic cell death by performing H_2O_2-mediated POCL.

The results of flow cytometry were further supported by western blot analysis of caspase-3 which is an apoptosis-regulating protein and also a member of the cysteine-aspartic acid protease family. The effects of CDNP on the expression of pro-apoptotic caspase-3 were shown in Figure 5(b). In comparison with control, no significant reduction in pro-caspase-3 expression was observed in HPOX, CA and PPIX group. CDNP consisting of HPOX and PPIX also showed negligible effects on the

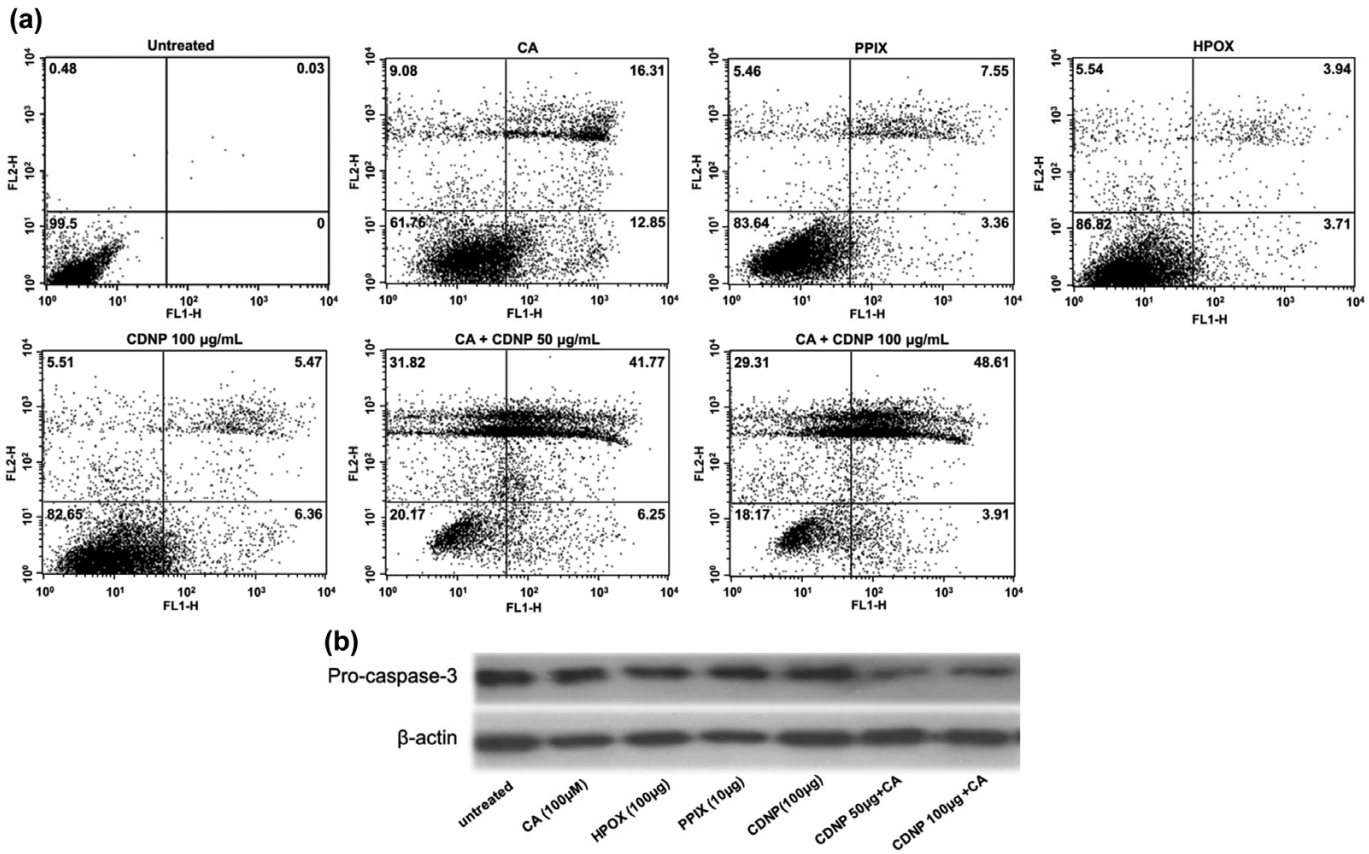

Figure 5. CDNP-mediated apoptotic cell death of SW620 cells. (a) Flow cytometric analysis of SW620 using Annexin V-FITC and PI. Cells were treated with a respective formulation for 24 h and a total of 1.0×10^4 cells stained with Annexin V-FITC and PI were counted for analysis. Data shown are representative of three independent experiments. (b) The effects of CDNP on the expression of apoptosis-related proteins. Data shown are representative of three independent experiments.

expression of pro-caspase-3. However, co-treatment of CDNP and CA significantly decreased the expression of pro-caspase-3, as expected from the results of cell viability assay (Figure 3(b)) and flow cytometry (Figure 5(a)). The observations suggest that pre-treatment with CA is required to generate a sufficient amount of H_2O_2 which could trigger degradation of peroxalate esters to generate toxic singlet oxygen in peroxalate chemiluminescence based chemi-dynamic anticancer therapy. There are various other possible ROS inducers which could be more potent than CA, which could increase the level of intracellular ROS in cancer cells. In future work, we could focus on screening of potent ROS inducers and possibly incorporate them in the polymer backbone, thereby enhancing the efficacy of CDT.

At present, PDT has been used to treat tumors on or just under the skin or on the outer lining of internal organs/cavities.[11] Penetration depth (~10 mm) of light limits the application of PDT in various solid tumors.[12] Interstitial insertion of cylindrical optical fibers to increase penetration depth of photons was proven to be effective, but it involves minor invasive procedures.[31] Based on our previous studies on H_2O_2-responsive polymers, we made an effort to exploit the energy released from H_2O_2-triggered POCL as a source to excite photosensitizers to generate $^1O_2$ and drive tumor cells to apoptosis. We successfully developed light-free photodynamic nanoparticles that could provoke cancer cell death by utilizing H_2O_2-triggered POCL without an external energy source. CDNP could take the photosensitizer-based cancer therapy to next paradigm by expanding the POCL energy transfer mechanism in treating cancers located at the deeper part of the body.

4. Conclusions

In summary, we have validated that CDNP could induce $^1O_2$ in the presence of H_2O_2, without any external energy source to exert highly potent anticancer activity. This work provides for the first time, a proof of concept for light-free PDT as a novel anticancer therapeutic system which exploits POCL as a light source. We anticipate that POCL-based chemi-dynamic nanoparticles could provide solutions to the challenges in PDT and hold great potential as a promising cancer treatment modality.

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