Apoferritin protein cages: a novel drug nanocarrier for photodynamic therapy†

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A methylene blue-encapsulated apoferritin complex shows cytotoxic effects on MCF-7 human breast adenocarcinoma cells when irradiated at the appropriate wavelength.

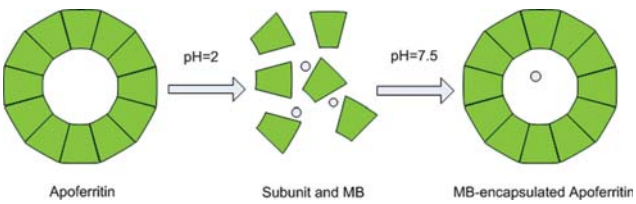
Photodynamic therapy (PDT) is a promising mode of treatment for both oncological (*e.g.*, tumors and dysplasias) and nononcological (*e.g.*, age-related macular degeneration, localized infection, and nonmalignant skin conditions) applications.[1–5] The PDT therapy involves the use of photochemical reactions mediated through the interaction of photosensitizing agents (or so-called photosensitizers), light, and oxygen. Optimal therapy requires the PDT drug molecule to be protected from degradation and reach its target cell and preferably its intracellular location. Efforts are currently underway to produce new generations of PDT drugs and novel nanocarriers (with size ranging from 1 to 100 nm) for their targeted delivery. The family of nanocarriers can be broadly categorized as polymer, lipid, surfactant and nanomaterial-based systems.[6] Compared to micrometre and sub-micrometre size carriers such as liposomes, nanocarriers provide higher surface area-to-volume ratio, and have the potential to increase solubility, enhance bioavailability, improve controlled release and enable precision targeting of the entrapped compounds to a greater extent. As a consequence of their improved stability and targeting, the amount of therapeutic molecules required to exert a specific effect when encapsulated in nanocarriers might be much less than the amount required when unencapsulated.[7,8]

In this study, we use apoferritin as a novel nanocarrier to load methylene blue (MB) and demonstrate its potential applications for photodynamic therapy of cancer. Apoferritin, the protein component of the iron-storage protein ferritin, consists of 24 symmetrically related subunits forming a near-spherical hollow shell of external diameter $\sim$13 nm and internal diameter 7.5 nm. Apoferritin has 14 channels, which are formed at subunit intersections with diameters of 3–4 Å and which connect the outside of the apoferritin molecule with its interior.[9–12] To the best of our knowledge, there is no previous report of PDT applications using this nanostructured protein as a nanocarrier.

Scheme 1 illustrates the process involved in the encapsulation of MB into apoferritin during its reassembly.‡ The protein cage of apoferritin can be disassociated into 24 subunits at low pH (2.0), and the subunits reconstitute into a complete structure in a high pH (7.5) environment. Methylene blue, due to its low toxicity and high quantum yield of singlet oxygen generation ($\Phi\Delta$ = 0.52), has been widely used as a photosensitizer.[13,14] However, the clinical use of MB has been hampered by its propensity for rapid chemical alteration when systematically applied: in biological environments, MB is thought to react with NADH/NADHP by accepting electrons, and the resulting leukomethylene blue (LMB) has negligible photodynamic activity.[15,16] The reassembly of apoferritin at higher pH (such as pH 7.5–8.0) facilitates the capture of MB.[17–20] MB is retained within the apoferritin shell because the molecule size (1 nm) is larger than the channel (3–4 Å). We determined that there was approximately one MB molecule in each apoferritin (see supplementary information†). The concentration of the resulting MB-encapsulated apoferritin complex under study was equivalent to 0.3 μM MB, which was coincidentally identical to the concentration level in MB-loaded polyacrylamide nanoparticles as reported previously.[21]

The production of singlet oxygen was monitored by the Singlet Oxygen Sensor Green reagent (SOSGR) (Invitrogen).[22] The fluorescence emission of SOSGR was recorded when excited at 376 nm. As demonstrated in Fig. 1, the fluorescence intensities continuously increased as the mixtures of SOSGR with two different MB formulations (*i.e.* 3 μM MB and a MB-loaded apoferritin complex with a concentration equivalent to 0.3 μM MB) were irradiated at 633 nm over a period of time, while the two controls (one containing only 5 μM SOSGR, and another one consisting of a mixture of 3 μM MB and 5 μM SOSGR without laser irradiation) showed negligible signal changes during the same time frame. It was evident that MB-encapsulated apoferritin complex generated a similar amount of $^1O_2$ in 35 min as 3 μM pure MB did in 8 min.

The cytotoxic activity of MB-loaded apoferritin was tested using a fluorescein diacetate (FDA)–propidium iodide (PI) two-color fluorescence viability assay on MCF-7 human



Scheme 1 Schematic representation of the encapsulation of methylene blue (MB) during the reassembly of apoferritin.

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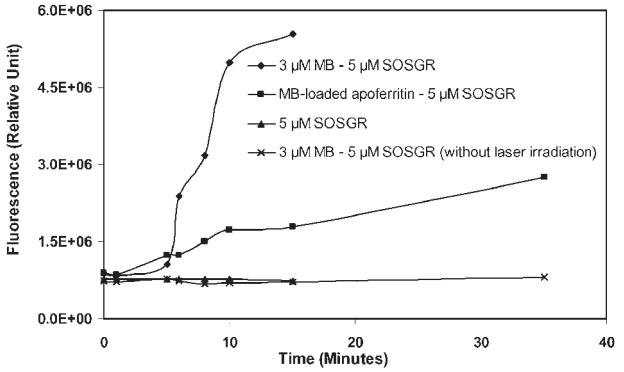

Fig. 1 Singlet oxygen production as monitored by Singlet Oxygen Sensor Green reagent (SOSGR). Two different MB formulations and two controls were compared.

breast adenocarcinoma cells. Fluorescein diacetate is a non-polar, non-fluorescent compound which can pass through the cell membrane whereupon intracellular esterases cleave off the diacetate group producing the highly fluorescent product fluorescein. Fluorescein accumulates in cells which possess intact membranes so its green fluorescence can be used as a marker of cell viability.[23] Propidium iodide is a non-permeant dye that can penetrate the membranes of dying/dead cells. It intercalates into the major groove of the DNA and produces a highly fluorescent adduct so non-viable cells can be identified by positive red fluorescence.[24] MCF-7 cells were grown on glass bottomed Petri dishes coated with poly-D-lysine. After removing the culture media, the Petri dish was rinsed carefully with PBS buffer. The cells were covered with 5 ml of culture medium and placed in the cell culture incubator at 37 °C. In a typical confocal experiment, the cells were incubated with MB-encapsulated apoferritin for 2 h, irradiated under the He–Ne laser (633 nm, 20 mW) for 30 min, and then exposed to FDA–PI for up to 2 h. Fluorescein generated by intracellular esterases of FDA was excited with a 488 nm argon laser and PI was excited with a 561 nm diode laser. Images were taken for up to 2 h to monitor cell death. As evidenced in Fig. 2, localized illumination induced death of only those cells located in the beam of light. The cytoplasm of the cultured cells lost its green fluorescence, in a time-dependent manner (see supplementary information†). Concomitant with the loss of FDA, the nuclei of the compromised cells were observed to increase their incorporation of PI, indicated by the red fluorescence (Fig. 2A–D). Whereas other cells, in contact with MB-loaded apoferritin but not directly illuminated by the laser, remained intact and viable, as indicated by the green fluorescence. For the MCF-7 cells, the amount of dead cells almost doubled from 1 h to 1.5 h, comparing Fig. 2A to 2C. These data suggest that it takes a certain amount of time for the photoinduced singlet oxygen to trigger the cell death. Two control experiments were carried out to verify the photocytotoxicity of the MB-loaded apoferritin complex (as shown in Fig. 3): one was performed without MB-loaded apoferritin but with red light irradiation under the same conditions (Fig. 3A); the other was carried out with MB-loaded apoferritin but without light exposure (Fig. 3B). Neither revealed significant cell death in the illumination area. Therefore, both light and MB-loaded

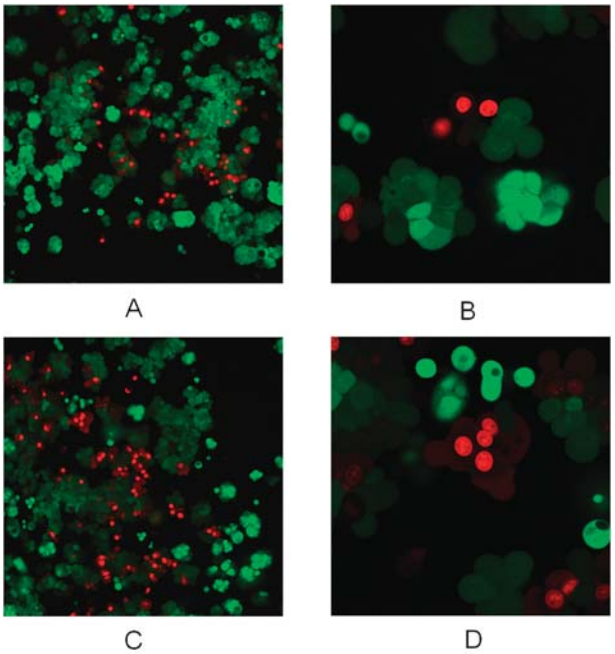

Fig. 2 Confocal images of MCF cells, loaded with MB-loaded apoferritin for 2 h, laser-irradiated 30 min, and exposed to FDA–PI for different amounts of time, showing a time-dependent response: 1 h (pictures A and B, taken with a 10× and a 40× oil immersion objective, respectively) and 1.5 h (pictures C and D, taken with a 10× and a 40× oil immersion objective, respectively). Fluorescence images of cells were captured on a Carl Zeiss LSM 510 confocal microscope equipped with multiple lasers (Carl Zeiss Inc, Thornwood, NY).

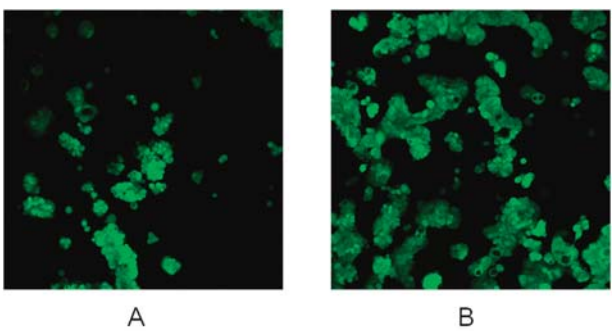

Fig. 3 (A) A confocal image of MCF-7 cells, which were exposed to 633 nm laser for 30 min in the absence of MB-loaded apoferritin, and subsequently exposed to FDA–PI for 1.5 h; and (B) a confocal image of MCF-7 cells, which were loaded with MB-loaded apoferritin for 2 h, and exposed to FDA–PI for 1.5 h (without being laser-irradiated directly). Both images were taken with a 10× objective.

apoferritin, in combination, are required to exert a cytotoxic effect on target cells.

Apoferritin is readily taken up by cells as suggested by previous studies on ferritin.[25] Further, exogenous apoferritin exposure has been shown to increase cellular ferritin content as detected by immunoblots from cultures that were treated with apoferritin.[26] We hypothesize that the MB-loaded apoferritin complex might be internalized into target cells, and hence delivered singlet oxygen intracellularly. In addition, as

encapsulated MB molecules were protected by the apoferritin cages, its inactivation *via* the reduction of the cation to the neutral leukomethylene blue was largely prevented.

In summary, we present here the synthesis and characterization of an apoferritin-based nanocarrier, which is designed to be internalized by tumor cells and deliver singlet oxygen intracellularly for PDT. As a model, we demonstrate the successful loading of methylene blue into apoferritin nanocarriers, and also show the positive effect of this complex on singlet oxygen production. Experiments are currently in progress to achieve higher payload per cage through the use of chemically- or genetically-engineered protein cages, and to confirm the intracellular uptake of fluorophore-tagged apoferritin by the tumor cells using techniques such as confocal microscopy.[27] Our preliminary *in vitro* PDT study using the MB-loaded apoferritin nanocages was conducted on MCF-7 cells with cytotoxic effects following irradiation as per photodynamic therapy protocols. The delivery of photosensitizers through targeted nanocarriers that could be internalized by cells provides an alternative route of delivery for photodynamic drugs into cells.

Notes and references

‡ Apoferritin from equine spleen was obtained from Sigma, and prepurified using a PD-10 column (17-0851-01, GE Health) before use. Its actual concentration after reassembly was determined using a BCA Protein Assay Kit from Thermo Scientific Pierce (Product No. 23225).

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