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Liposomal Surface-Loading of Water-Soluble Cationic Iron(III) Porphyrins as Anticancer Drugs

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Abstract: A novel design of anticancer drug delivery system, based on an electrostatic binding of negatively charged liposomes and cationic metalloporphyrins under physiological conditions, is reported. A lack of cytotoxicity of the iron(III) porphyrin-loaded liposomes and an efficient generation of a toxic hydroxyl radical ($OH^{\bullet}$) from a superoxide anion radical ($O_2^{-\bullet}$) through the iron(III)-catalyzed dismutation and the Fenton-like reaction allow for a targeted necrosis of tumor cells where the concentration of $O_2^{-\bullet}$ is locally increased as a result of the reduced activity of superoxide dismutase and catalase in these cells.

Keywords: Anticancer drug delivery system; liposome; cationic metalloporphyrins; superoxide anion radical; hydroxyl radical

Herein we report a novel design of anticancer drug delivery system, based on an electrostatic binding of negatively charged liposomes and cationic metalloporphyrins under physiological conditions. A lack of cytotoxicity of the iron(III) porphyrin-loaded liposomes and an efficient generation of a toxic hydroxyl radical ($OH^{\bullet}$) from a superoxide anion radical ($O_2^{-\bullet}$) through the iron(III)-catalyzed dismutation and the Fenton-like reaction allow for a targeted necrosis of tumor cells where the concentration of $O_2^{-\bullet}$ is locally increased as a result of the reduced activity of superoxide dismutase (SOD) and catalase in these cells. Here we focus on the novel design of the liposome and in vitro tests using tumor cells to investigate destruction of cancer cell; detailed studies on the structure of the porphyrin-loaded liposomes and in vivo tests are outside the scope of the present study.

Cationic porphyrins continue to be the focus of a great deal of attention because of the promise they have for use in photodynamic,[1] antiviral,[2] and anticancer[3,4] therapies. The cationic chromophores 5,10,15,20-tetra(*N*-methylpyridinium-*x*-yl)porphyrins (H_2T*x*MPyP, $x = 2-4$) are water soluble and have a Coulombic attraction for DNA.[5,6] These porphyrins appear to enter cells via pinocytosis[7] and tend to accumulate in mitochondria.[8,9] While potential therapeutic and clinical applications of porphyrins abound, they are known to undergo widespread biodistribution to both tumor and nontumor sites, with the latter frequently resulting in notable

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toxicities to healthy tissues.[10] Attenuation of such toxicities and tumor targeting are the proven benefits of liposome-loaded drug formulations.[11,12] Indeed, Maruyama et al. have reported that introduction of poly(ethylene glycol)-coupled transferrin to liposomes can successfully optimize tumor-targeting and reduce premature drug leakage from the particle.[13] In this report, we describe that stearate-containing liposomes act as nanocarriers for cationic iron(III) porphyrins. We compare the interactions of two cationic water-soluble porphyrins Fe^{III}T*x*MPyP (*x* = 2, 4). In their presence, the liposome has been found to kill tumor cells through the production of reactive oxygen species without photoirradiation. Alamar-blue assay reveals that the two porphyrins vary dramatically in their abilities to kill tumor cells.

While free radicals are proposed to be the key species responsible for DNA damage, $O_2^{-\bullet}$ generated in vivo has only moderate biological activity, mainly because of its negative charge. Unless there is an anion channel available, $O_2^{-\bullet}$ can only very slowly cross biological membranes. Nevertheless, chemical, enzymatic, or phagocytic generation of $O_2^{-\bullet}$ has been observed to cause considerable biological damage. Superoxide might be converted spontaneously or enzymatically to H_2O_2 by dismutation. The latter oxidizing agent would then be able to migrate within the cells and generate the $OH^\bullet$ radical. Both in vivo and in vitro studies have shown that iron complexes catalyze the decomposition of generated H_2O_2 within cells, resulting in the production of the oxidative $OH^\bullet$ radical.[14] This is known as the Fenton reaction and represents a source of the $OH^\bullet$ radical. A version of the iron-catalyzed dismutation of $O_2^{-\bullet}$ followed by the Fenton reaction adapted to the current situation is (i) $LFe^{III} + O_2^{-\bullet} \rightarrow LFe^{II} + O_2$, (ii) $LFe^{II} + O_2^{-\bullet} + 2H^+ \rightarrow LFe^{III} + H_2O_2$, and (iii) $LFe^{II} + H_2O_2 \rightarrow LFe^{III} + OH^\bullet + OH^-$.

It has been reported that introduction of a cationic *meso*-(*N*-methylpyridinium-2-yl) group greatly enhance an SOD activity of a manganese(III) porphyrin as a typical SOD mimic, which suggests that the species responsible for attack on the porphyrin is anionic in nature as a result of the electrostatic interaction with the cationic charge on the porphyrin periphery.[15] We anticipate that similar electrostatic

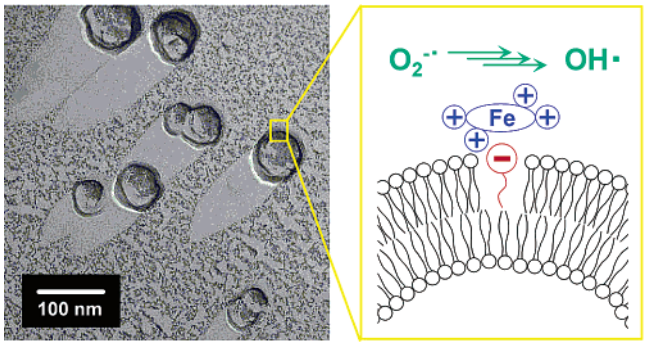

Figure 1. TEM picture of the porphyrin-loaded liposome **1** showing monodispersed particles with an average hydrodynamic diameter of 30 nm from dynamic light scattering measurements.

effects apply to the iron-catalyzed generation of $OH^\bullet$ from $O_2^{-\bullet}$. However, cationic porphyrins are water soluble and therefore readily pass through glomeruli. To overcome this difficulty, we used liposomes as nanocarriers retentive of cationic porphyrins. The iron(III) porphyrin-loaded liposome (**1**) was prepared by an ultrasonic irradiation of an aqueous solution containing L-α-phosphatidylcholine dimyristoyl (DMPC) (200 μmol), sodium stearate (SA_{Na}) (4 μmol), and $[Fe^{III}Br(T2MPyP)]^{4+}$ tosylate (1 μmol) at 5 °C for 30 min, followed by incubation at room temperature for 1 h and filtration with a 0.22 μm pore sterilized filter. Figure 1 shows the resulting porphyrin-doped nanoparticles, which are spherical, highly dispersed ($\phi_{av} \approx 30$ nm), and stable in H_2O and phosphate-buffered saline for 1 week at 10 °C and for 2 days at 37 °C ($T_c = 25$ °C). In particular, the size of the liposomes permits their extravasation and accumulation in a variety of pathological sites, where the permeability of the vascular endothelium is increased, such as infarct zones and tumors. This fact provides at least an opportunity of physiology-based targeting of porphyrin-loaded liposomes to these pathological areas via the passive targeting effect. Such drug-doped carriers are preferentially taken up by tumor tissues by virtue of the enhanced permeability and retention effect. The enhanced permeability is the property of such tissues to engulf and retain circulating liposomes owing to their leaky vasculature.

While a hydrophobic site of a liposomal bilayer can accommodate lipophilic drugs, an aqueous compartment encapsulated in liposomes can contain hydrophilic ones. We found that Fe^{III}T*x*MPyP can be electrostatically immobilized at the outer (and probably inner) *surface* of liposomes (vide infra), and that the surface-loading is advantageous over the conventional core-loading to generate $OH^\bullet$ from $O_2^{-\bullet}$ that diffuse in from the outer phase. Our unique design of the liposome accommodating porphyrins at the surface also affords promise for the transferrin-free and porphyrin-based tumor targeting.[13]

Figure 2 shows in vitro tests demonstrating the effect of **1** on the viability of Lewis lung carcinoma (LLC) tumor cells.

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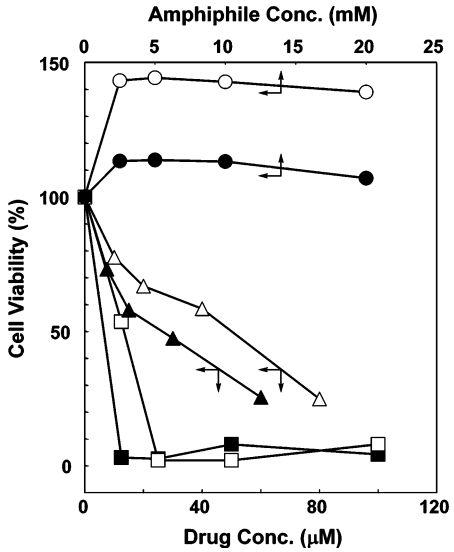

Figure 2. Cell viability tests of LLC tumor cells using Alamar-blue assay for the DMPC/SA_{Na} (50:1) liposomes in the absence (○) and the presence of FeT2MPyP (□) (**1**), and the pH-sensitive liposomes DMPC/DTDAB/OA_{Na}/Tween-80 (90:5:5:1) in the absence (●) and the presence of FeT2MPyP (■) (**2**), cisplatin (△), and MMC (▲). The porphyrin concentration (in μM) was determined from the amount of the amphiphile (in mM), assuming that [FeT2MPyP]/[amphiphile] = 1/200.

Significant damage to such impregnated tumor cells was observed, as shown by open-square plots. The porphyrin concentration at which half of the cells survive (IC_{50}) was only 13 μM for **1**. The lack of cytotoxicity was shown in a control experiment using Mn^{III}T2MPyP in place of Fe^{III}-T2MPyP, which suggested that the cell damage was induced by the $OH^{\bullet}$ radical, considering that MnT2MPyP only showed an SOD activity but did not participate in the Fenton reaction.[14,15] Added support for this interpretation was provided by ESR experiments which allowed the in vitro detection of the $OH^{\bullet}$ radical generated from $O_2^{-\bullet}$ in the presence of FeT2MPyP.[14] The $OH^{\bullet}$ radical was trapped by 5,5-dimethyl-1-pyrroline 1-oxide to show a four-line ESR signal with a hyperfine splitting constant of 14.9 G. Furthermore, control experiments in the presence of scavengers such as SOD and MnT2MPyP revealed that the cell damage caused by **1** was successfully canceled by these scavengers (IC_{50} > 100 μM). Interestingly, replacement of FeT2MPyP with FeT4MPyP reduced the cytotoxicity. This is reminiscent of the lower SOD activity of MnT4MPyP than MnT2MPyP due to the lower local charge density near the reaction site, indicating that electrostatic attraction for $O_2^{-\bullet}$ is a dominant factor. The cell viability test also revealed that the porphyrin in **1** had a higher cytotoxicity than mitomycin c (MMC) and cisplatin, which showed IC_{50} values of 26 μM and 50 μM, respectively.

Electrokinetic potential measurements, in conjunction with spectroscopic techniques, revealed the nature of the electrostatic binding. The negative ζ potential of the DMPC/SA_{Na} liposome (−30 mV) shifted positively to +25 mV and +23 mV upon the loading of FeT2MPyP and FeT4MPyP, respectively. The emission from the DMPC/SA_{Na}/H_2T2MPyP liposome in H_2O showed a maximum at 642 nm, which was close to that from a 2-propanol solution of H_2T2MPyP alone (λ_{em} = 643 nm) rather than that from an aqueous solution (λ_{em} = 635 nm), indicating that H_2T2MPyP was efficiently introduced into the liposome and surrounded by a relatively hydrophilic environment in the liposome. The GPC elution curve (Toyopal HW-65S) for **1** using H_2O as an eluent, detected at 230 nm for DMPC, was almost identical to that detected at 409 nm for FeT2MPyP, indicating that the electrostatic binding was substantial. On the other hand, the DMPC liposome without SA_{Na} lacked negative charges and did not load cationic porphyrins at the surface. In this case, a much lower cytotoxicity was observed by the core-loaded FeT2MPyP. Thus, the potential of using negatively charged liposomes as drug carriers for water-soluble positively charged porphyrins has been demonstrated.

Another curious issue is an enhanced cell damage caused by an FeT2MPyP-loaded pH-sensitive liposome (**2**) as shown by closed squares in Figure 2 (IC_{50} = ca. 6.5 μM). The liposome was composed of DMPC, dimethylditetradecylammonium bromide (DTDAB), and Tween-80 to which was added sodium oleate (OA_{Na}) for electrostatic binding of FeT2MPyP. We found that the DMPC/DTDAB/OA_{Na}/Tween-80 liposome with a composition of 90:5:5:1 was highly fusion competed when exposed to an acidic environment of pH < 5.5 and yet showed no decomposition or cytotoxicity at a physiological pH as shown by the closed circles in Figure 2. At a lower pH to be encountered locally within the tumor cells, the pH sensitivity manifests itself and decomposition occurs. This triggers a rapid and convenient nonenzymatic decomposition process that releases FeT2MPyP from the DMPC/DTDAB/OA_{Na}/Tween-80/FeT2MPyP liposome (**2**). Preliminary experiments using human umbilical vein endothelial cell (HUVEC) revealed that the porphyrin in **2** was less toxic for HUVEC (IC_{50} = ca. 14 μM) than for LLC, while cisplatin (31 μM) and MMC (11 μM) were equally or even more toxic for HUVEC. Although the porphyrin in **2** was more toxic than cisplatin and MMC, a greater selectivity of action to tumor cells was indicated.

In conclusion we have demonstrated that liposome-loaded cationic porphyrins present a potential target for the design and development of novel anticancer drugs. The strong electrostatic interaction suggests that the porphyrin-loaded liposome could remain intact even in vivo, which is the topic of our future investigation. Our approach opens the door for liposomal loading and tumor-targeting of a much expanded selection of porphyrins.

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Supporting Information Available: Magnified image of the TEM picture in Figure 1 and a dynamic light scattering histogram of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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