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In order for patients to avail the therapeutic benefits of antioxidant drugs efficiently and conveniently, a robust oral delivery system needs to be developed. However, a common obstacle to oral drug delivery is ensuring that the drug remains functionally intact even after it has passed through the acidic environment of the gastrointestinal (GI) tract. To protect drugs within the GI environment, we formulated a design based on encapsulating liposomal drugs by using an alginate matrix as a carrier. The liposomal drug was composed of manganese porphyrin (Mn-por), which has been developed as a mimic of superoxide dismutase (SOD), as the therapeutic agent based on antioxidative effect, namely superoxide ($O_2{\cdot}^-$) inhibitory activity. Cytochrome *c* assay revealed that $O_2{\cdot}^-$ inhibitory activity of Mn-por could be maintained even after treatment of simulated gastric and intestinal fluids. We demonstrated that oral administration of the formulated drug significantly inhibited the growth of transplanted tumors in mice. The drug formulation presented in this study would be a good candidate for orally available system, which can effectively deliver SOD mimics.

Introduction

Excess production of reactive oxygen species (ROS) in the body are a cause of a broad spectrum of diseases (e.g., cancer, neurodegenerative diseases).[1] Superoxide dismutase (SOD) is a biological enzyme playing a key role in maintaining appropriate levels of ROS in eukaryotic cells. Superoxide anion ($O_2{\cdot}^-$) is the first species that initiates subsequent reaction producing more toxic ROS.[2] It has been shown that iron and manganese porphyrins have $O_2{\cdot}^-$ inhibitory activity.[3–7] As a mimic of SOD, metalloporphyrins have a great potential for treatment of ROS-related impairments.[8–10] To date, our group has designed porphyrin-based anticancer drugs and shown the enhanced drug efficacy by using pH-sensitive liposomes as drug carrier.[11–13] In this way, metalloporphyrins can effectively work as anticancer agent, but practical way of administrating them has been undeveloped. Although Kawakami *et al* reported *in vivo* therapeutic effect of manganese porphyrins administrated by intraperitoneal injection,[14,15] there still remains room for developing ways to administrate metalloporphyrins as SOD mimics. "Injection" as a common way to administrate drugs has an unfriendly aspect for patients because it requires visiting a therapeutic facility and inflicting a physical pain on patients. Development of an appropriate formulation for oral administration of SOD mimic is important in terms of improving quality of life of patients. In this study, we suggest an appropriate drug formulation for oral administration of manganese porphyrin, which was designed as SOD mimic (Fig. 1).

For a successful oral delivery, drugs must be protected against exposure to acidic pH and digestive enzymes in the gastrointestinal (GI) tract, and be adsorbed from intestinal region. Especially, there is concern about alternation of chemical structure of manganese porphyrins (detachment of Mn(II) from porphyrin ring) by exposure them to acidic environment in stomach. Since existence of Mn(II) in porphyrin ring is essential to exert its $O_2{\cdot}^-$ inhibitory activity,[6] the alternation of the chemical structure going through GI tract must be prevent for a substantial therapeutic effect in the oral administration. To date, encapsulating drugs in polymer-based carrier matrices such as pectin,[16] alginate,[17] and biodegradable polymer[18] have been used to protect the drugs from harsh physiological environments. In this study, calcium alginate hydrogel was used as a carrier matrix (Fig. 1, Box 1) because of its biocompatibility (as approved by the U.S. Food and Drug Administration) and sensitivity to pH. When alginates are exposed to an acidic aqueous solution, its carboxylate groups are protonated, rendering it insoluble in the acidic aqueous solution.[19,20] When alginate based drugs are located in gastric environment with acidic pH, the insoluble nature of the alginate matrix would be contribute to retention of embedded drugs in the alginate matrix. Moreover, enhancing hydrophobicity of

alginates in response to acidic environment may prevent influx of gastric fluid, resulting in suppression of altering the chemical structure of Mn-por, which is a cause of reducing $O_2^{\cdot -}$ inhibitory activity of Mn-por due to detachment of Mn(II).[6] Conversely, when the alginate is immersed in a basic or neutral aqueous solution such as intestinal environment, its carboxylate groups are deprotonated. This results in solubilization

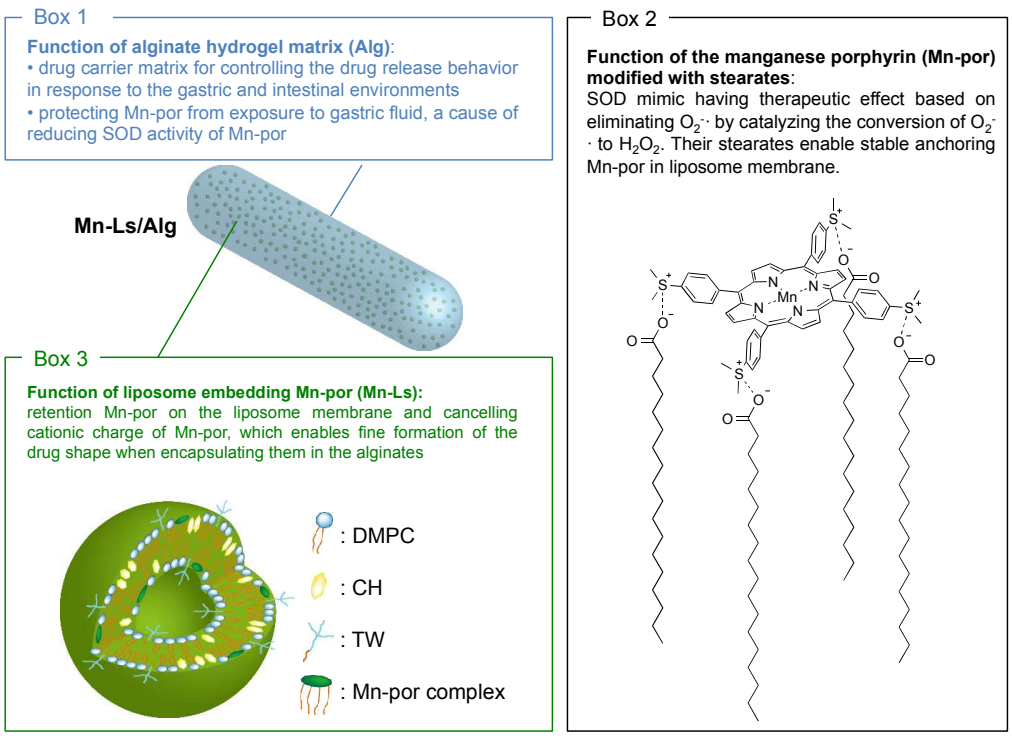

Figure 1. Drug formulation for oral administration of SOD mimic (abbreviated as Mn-Ls/Alg). Mn-Ls/Alg consists of alginate hydrogel matrix (Alg) encapsulating liposomes with embedded manganese porphyrin (Mn-Ls). Functions of each component are described in Box 1–3.

of the alginate matrix in intestinal environment, inducing release of embedded drugs from the matrix. In these respects, alginate is a suitable candidate for use as an oral carrier matrix.

In this study, we used a manganese porphyrin (Mn-por) bearing dimethyl(phenyl)sulfonium groups as SOD mimic. Its substantial $O_2^{\cdot -}$ inhibitory activity has been confirmed in previous study.[21,22] However, cationic nature of the Mn-por is unfavorable to form proper shape of drug for oral administration. In preliminary experiment, it was observed that alginate polymers were electrostatically crosslinked by sulfonium groups of Mn-por, and gelated soon after mixing together. This limited forming proper shape of drug (e.g., spheres, fibers). To avoid this obstacle, we modified Mn-por with stearates (Fig. 1, Box 2) and embedded them in liposomes (Fig. 1, Box 3). Modification of Mn-por with stearates allows for canceling cationic charge of Mn-por, and enables Mn-por to stably retain in the liposome because the long acyl chains of the stearates can anchor on lipid bilayer membrane of liposome.[23] The liposomes embedding Mn-por (Mn-Ls) can stably disperse in sodium alginate solution without gelation of alginates, contributing to fine shape forming of drug.

The aim of this study was to develop a robust system for the effective oral delivery of a SOD mimic. First, we describe the formulation and stability of the delivery vehicle for Mn-por, named Mn-Ls/Alg. Second, *in vivo* antitumor efficacy of orally administered Mn-Ls/Alg was evaluated using mice transplanted with cancer cells.

Materials and Methods

Materials

Synthesis procedure of Mn-por was shown in supplementary data (ESI). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from NOF corporation (Tokyo, Japan). Cholesterol (CH), Tween-20 (TW), sodium stearate, and sodium alginate (Alg) were purchased from Wako Pure Chemical (Osaka, Japan). A mouse rectal cancer cell line (Colon-26) was provided by RIKEN Bio-Resource Center (Tsukuba, Japan). Colon-26 cells were seeded onto a conventional cell culture dish and cultured in RPMI-1640 medium (189-02025, Wako) supplemented with 10% fetal bovine serum (FBS, Sigma), 10^5U/L penicillin (Sigma), and 100 mg/L streptomycin (Sigma). Cells were then incubated at 37°C in a humidified incubator with a 5% CO_2 atmosphere. Subconfluent cell cultures were passaged after treatment with 0.25% trypsin (T4049, Sigma) to dissociate the cells from the culture vessel.

Preparation of liposomes embedding manganese porphyrin (Mn-Ls)

Liposomes embedding Mn-por (Mn-Ls) were prepared as previously described.[13] Briefly, Mn-por (1 μmol) and sodium stearate (4 μmol) were added in a round-bottom plastic vial and dissolved in methanol to form complexes, which allowed for efficient incorporation of Mn-por into the liposome bilayer. Then, the other components (DMPC:CH:TW:Mn-por complex= 288:72:40:10, μmol) were added to the methanol solution. For preparing drugs containing Mn-por with higher concentration, 15 μmol of Mn-por was employed. The methanol solution was slowly evaporated on a rotary evaporator under reduced pressure to form a uniformly thin lipid film on the bottom of the vial. The dried lipid film was completely hydrated with 5 mL of distilled water at 45°C (above temperature of the transition temperature (T_c) of DMPC) for 0.5 h. The hydrated lipid film was vigorously stirred with a probe-type sonicator (30 W) until the appearance of the suspension changed from milky white to hazy transparent. Encapsulation efficiency of Mn-por in the liposomes was determined by a typical gel filtration column chromatography as described in supplementary data (ESI).

Measurement of size distribution of Mn-Ls

To determine the size distribution of liposomes, dynamic light scattering (DLS) measurements were performed using Nicomp 380 (Particle Sizing Systems, CA, USA) equipped with a helium-neon (He-Ne) laser (5 mW) operating at a wavelength of 632.8 nm. The scattered light was collected at a 90° angle at 37°C. All sample suspensions were filtered through 0.2-μm pore syringe filters before analysis. The polydispersity index (PDI) was used to evaluate broadness of size distribution, and calculated by the equation, $[(SD/mean)^2]$.

Freeze-fracture transmission electron microscopy (FF-TEM)

Liposome dispersions were frozen rapidly in liquid nitrogen and fractured using a cold knife (FR-7000A, Hitachi High-Technologies, Tokyo, Japan). To prepare the liposome replica, first, platinum vapor was exposed to the cross section of the frozen dispersion, and second, the cross section was treated with carbon vapor to build up the replica. Liposomes on the replica were washed out with methanol and distilled water, after which the replica was transferred onto a cupper grid. The liposome replica was visualized using a transmission electron microscope (H-7650, Hitachi High-Technologies) with 120 kV of acceleration voltage at −160°C.

Preparation of alginate matrix embedding Mn-Ls (Mn-Ls/Alg)

The Mn-Ls dispersion was mixed with equivalent volume of 2 wt% sodium alginate aqueous solution. Then, the mixture was injected into an aqueous solution of 1.8 M calcium chloride ($CaCl_2$) by using a syringe with a 27-gauge needle. The injection rate was set at 100 μL/min by using a micro-syringe pump. We determined overall encapsulation efficiency of Mn-por in the alginate matrix as described in supplementary data (ESI).

Field emission-scanning electron microscopy (FE-SEM)

The interior network structure of the alginate hydrogel matrix was observed using a field emission-scanning electron microscope (FE-SEM). Lyophilized Mn-Ls/Alg was fractured with a spatula to expose the interior network of the matrix. The fractured Mn-Ls/Alg was sputter-deposited with platinum, and then observed by FE-SEM (JSM-7600F, JEOL) with 20.0 kV of acceleration voltage.

Release behavior of Mn-por from the alginate matrix

To simulate the GI environment, acidic (pH 1.4) and mildly basic (pH 6.8) aqueous solutions were prepared to simulate gastric and intestinal fluids, respectively. The gastric fluid was composed of 24 mM HCl and 34 mM NaCl, and the intestinal fluid was composed of 23.6 mM NaOH and 1.18 mM KH_2PO_4. Mn-Ls/Alg were then immersed into the simulated gastric fluid and subsequently added to the intestinal fluid. In each process, fluid containing Mn-Ls/Alg was stirred at 70 rpm for 1 h at 37°C. In each case, the tolerance of Mn-Ls/Alg was evaluated by visual observation to confirm whether Mn-Ls/Alg was solubilized. Released amount of Mn-por from the alginate matrix was determined by measuring absorbance of supernatant of gastric or intestinal fluid including Mn-Ls/Alg. The absorbance was monitored at ~490 nm (the Soret band of Mn-por).

Evaluation of the integrity of Mn-Ls and Mn-por after digestion of alginate matrix

After exposure to the simulated physiological fluids, Mn-Ls were collected by centrifugation of the fluid at 10,000 rpm for 40 min. The supernatant was then filtered through a 0.22-μm pore filter. The microscopic structure of the collected Mn-Ls was observed with FF-TEM. The size distribution of the collected Mn-Ls was evaluated using DLS measurement. To confirm $O_2^{\cdot -}$ inhibitory activity of Mn-Ls after treatment with simulated GI fluids, conventional cytochrome *c* assay[2,24] was carried out (ESI).

Evaluation of *in vivo* antitumor efficacy of Mn-Ls/Alg

Female BALB/cSlc mice (12 weeks for age) were purchased from Sankyo Labo Service (Tokyo, Japan), were kept for 1 week at animal facility and used at 13 weeks of age. They were housed in wire-mesh cages at a temperature of 20 ± 2℃ and relative humidity of 55 ± 5% under a 12 h light-dark cycle, and were allowed free access to tap water and experimental normal diet. All *in vivo* tests were performed according to the Guide Principles for the Care and Use of Laboratory Animals of Japanese Pharmacological Society and in compliance with the guidelines regarding animal experiments of the Tokyo University of Science's Institutional Animal Care and Use Committee. To evaluate the antitumor efficacy of orally administered Mn-Ls/Alg *in vivo*, the 13-week-old mice were used. Mn-Ls/Alg fibers were cut into pieces to ease oral administration. Mice were orally administered 0.4 mL of Mn-Ls/Alg containing 30 μM of Mn-por. The concentrations of Mn-por in Mn-Ls/Alg were calculated from initial concentration of Mn-por in sodium alginate solution (1.0 mM

or 1.5 mM) and overall encapsulation efficiency (2%). The control group was administrated 0.4 mL of PBS instead of Mn-Ls/Alg. Each group consisted of 5 animals. Four hours after the oral administration of Mn-Ls/Alg, Colon-26 cells (5×10^6 cells in 0.1 mL of PBS) were transplanted into the back of the mice. The Mn-Ls/Alg was administered at 24-h intervals. The dimensions of the transplanted tumor were measured 8 days after the first dose of the drug was orally administered. The tumor volume (V) was calculated using the formula for a semi-ellipsoid: $V = (4/3)\pi abc$, where a, b, and c are minor radius, major radius, and height of the transplanted tumor, respectively.

Results

Formulation of the Mn-Ls/Alg

In our previous studies, we developed several types of phospholipid liposomes containing porphyrin complexes embedded in the bilayer

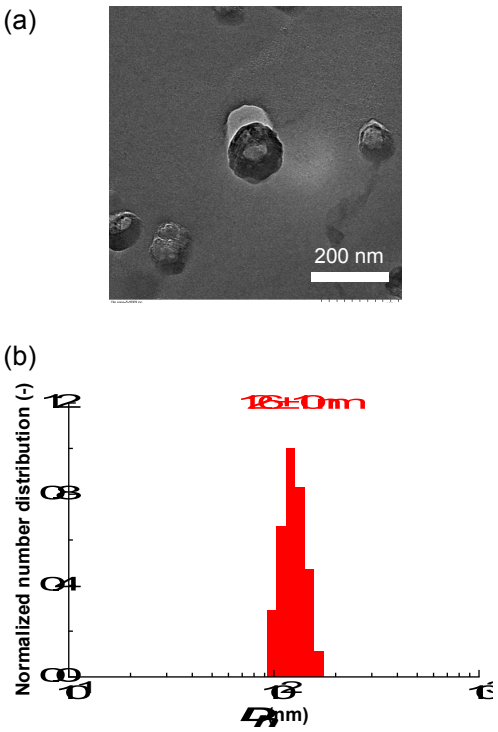

Figure 2. (a) FF-TEM image of Mn-Ls prepared by sonication (b) The size distribution of the Mn-Ls as determined by DLS measurement in deionized water at 37°C. Values are expressed as mean ± SD. PDI =0.01.

membrane by using a sonication method.[11–13] In the present study, liposomes containing Mn-por (Mn-Ls) were prepared by sonication, as previously reported. The successful preparation of Mn-Ls was confirmed by FF-TEM and measurement of DLS. FF-TEM revealed that the shape of the resulting Mn-Ls was a spherical with diameters that ranged from 100 nm to 140 nm (Fig. 2a); the Mn-Ls was found to be individually dispersed and had not aggregated. The mean diameter of the Mn-Ls was 126 nm, as measured by DLS, and the size distribution was monomodal with a small range (S.D.= 10 nm) (Fig. 2b). The diameter of the Mn-Ls prepared in the present study was larger than that of the liposomes prepared in our previous studies (20–50 nm).[11–13] The reason for this increment of the diameter may be that cholesterols were embedded in the liposome. Generally, it is known that diameter of liposome increase with the increasing the amount of cholesterol in the liposome.[25] Together, these findings show that the conventional sonication method used in the present liposomes with uniform dispersibility.

To maintain therapeutic function of the drugs, Mn-Ls were encapsulated in alginate hydrogel fibers to protect them from harsh environment of the GI tract. The alginate hydrogel fibers formed soon after the alginate was discharged into the $CaCl_2$ aqueous solution. The color of the resulting alginate fibers was greenish yellow, indicating that Mn-Ls was encapsulated within the alginate fibers (Fig. 3a). The mean diameter of alginate fibers was approximately 100 μm (Fig. 3b).

The inner structure of the carrier matrix is an important factor in the release of drugs and in the response of the carrier matrix to the external environment. In the present study, the inner structure of the alginate matrix was investigated using FE-SEM. FE-SEM revealed that the alginate matrix had a hierarchical structure. At low magnification, pores measuring a few hundreds micrometers were observed in the alginate matrix (Fig. 3c). At high magnification, submicron-sized pores were observed, distributed uniformly in the walls of larger pores (Fig. 3d). This porous structure may allow a solution from the external environment to infuse into the matrix, thereby ensuring a rapid response to changes in the external environment.

Encapsulation efficiencies of Mn-por in each encapsulation step were shown in Table 1. Encapsulation efficiency of Mn-por in the liposomes was 71%. This value was relatively high compared to that encapsulating hydrophilic drugs.[26] It has been known that encapsulation efficiency of hydrophobic drugs in liposomes are higher than that of hydrophilic drugs.[27] In the present study, the hydrophobic alkyl chains may enable Mn-por to stably anchor on the liposome membrane and contribute to its higher encapsulation efficiency. However, encapsulation efficiency of Mn-por in the alginate matrix was very low (3%). Indeed, leakage of Mn-Ls from the matrix was observed in the $CaCl_2$ aqueous solution after the encapsulation (data not shown). As a result, overall encapsulation efficiency was lowered (2%). Leakage of Mn-Ls after encapsulating them in the alginate matrix may be due to the larger pores in the alginate matrix (Fig. 3c).

Response of Mn-Ls/Alg to a simulated gastric and intestinal environment

For the successful oral delivery of therapeutic drugs to the intestine, the carrier matrix transporting the drugs needs to respond appropriately to both gastric and intestinal environments. Therefore, the matrix needs to be insoluble in the gastric fluid to protect the encapsulated drug and soluble in the intestinal fluid to permit the release of the drugs. Figure 4 demonstrated that the response of the Mn-Ls/Alg in the simulated gastric or intestinal fluid. The alginate matrix of Mn-

Ls/Alg was insoluble in the simulated gastric acid (Fig. 4a). In contrast, the alginate matrix was rapidly dissolved within a few minutes of immersion in the simulated intestinal fluid (Fig. 4b). Moreover, it is revealed that the drug release from alginate matrix exhibits favourable behavior in response to each GI fluid (Fig. 4c). In the simulated gastric fluid, the released amount of the drugs remained at very low levels (~1%) for 6.8 h (Fig. 4c, open circles). It was also confirmed that more drug release did not occur even after one day. On the other hand, in the simulated intestinal fluid, the drugs were rapidly released from the alginate matrix. The released amount reached to ~80% at 1 h (Fig. 4c, filled circles). The level of released amount in the simulated intestinal fluid was unchanged even after several days. As seen in this study, the favorable response of the alginate matrix to the simulated GI fluids is due to the pH-sensitivity of alginates. Since the p*Ka* value of alginates is approximately 3.5 [20] when exposed to acidic fluids, such

Table 1. Encapsulation efficiency of Mn-por each encapsulation step

Encapsulation step	Encapsulation efficiency of Mn-por (%)
Mn-por into the liposomes	71
Mn-Ls into the alginate matrix	3
Overall	2

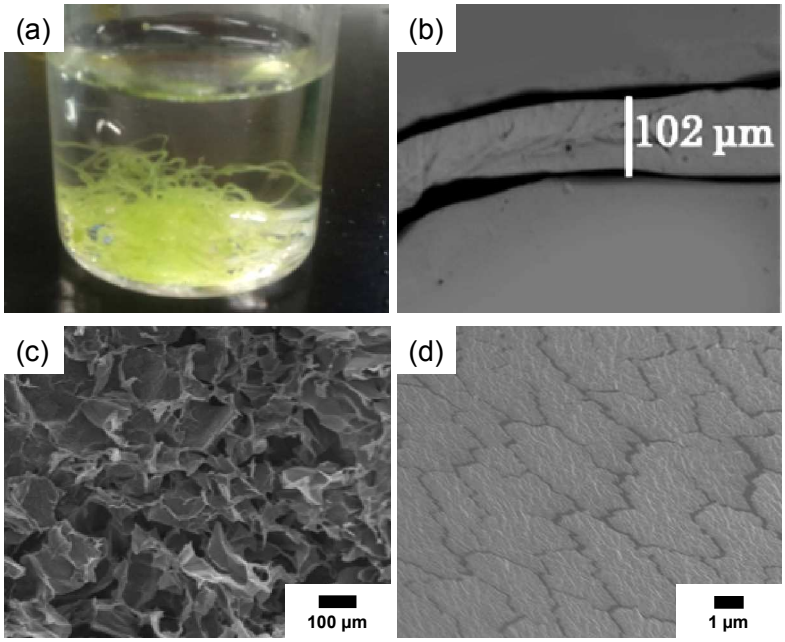

Figure 3. Structural details of prepared Mn-Ls/Alg. (a) Photographic image of Mn-Ls/Alg immediately after preparation. (c) FE-SEM image of lyophilized Mn-Ls/Alg. (d) FE-SEM image that magnified surface of the pleated part of (c).

as the simulated gastric fluid (pH 1.4) used in this study, the carboxylate anions of the alginates convert into carboxyl groups. Due to the lack of negatively charged functional groups, alginates are insoluble in acid aqueous fluids. In addition, intermolecular association of the alginate polymer chains, such as hydrogen bonding and polymer chain entanglement, may also contribute to the maintenance of the insoluble state. These properties of the alginate matrix would limit influx of water, contributing to further drug leakage from the matrix (Fig. 4, open circles). In the *in vivo* gastric environment (pH 1.5–3.5),[23] Mn-Ls/Alg is expected to pass through without solubilization of the carrier matrix and leakage of the drugs. Considering the p*Ka* of the alginate, the carboxyl groups of the alginates are likely to have been deprotonated in the simulated intestinal fluid (pH 6.8). It is possible that the increase of negatively charged carboxylate groups in the alginate matrix mediated the hydration of the matrix, thereby causing the dilution of positively charged species such as Ca^{2+}, which enable the alginate to crosslink. The sequence of these events should be related to the solubilization of the alginate. The results shown in this study (Fig. 4b and c, filled circles) indicate that the Mn-Ls/Alg matrix can be solubilized, facilitating the quickly subsequent release of Mn-Ls. The Mn-Ls/Alg may show similar solubilization and drug release behavior *in vivo* because the pH of the human intestine ranges from 5.5 to 8.6.[23] However, it was also found that ~20% of the drug was remained in the matrix in the simulated intestinal fluid (Fig. 4c, filled circle). One possible reason for the residual of drugs is that Mn-Ls may be physically adsorbed on the alginate matrix.

Morphological integrity and $O_2^{\cdot -}$ inhibitory activity of Mn-Ls after digestion of the alginate matrix

For a drug to exert its therapeutic effect within the target tissue, the drug must remain functionally intact after passing through the GI

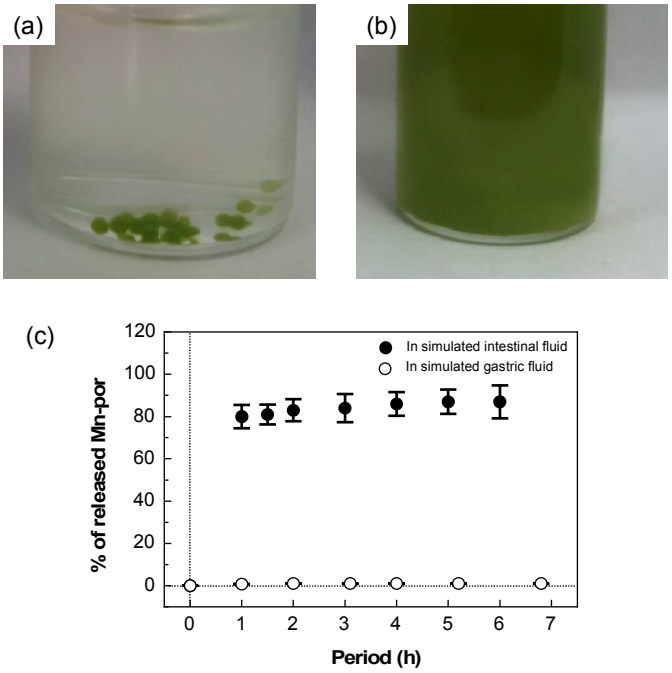

Figure 4. Response of Mn-Ls/Alg to simulated GI fluids. Photographic image of Mn-Ls/Alg in simulated gastric fluid (a), and intestinal fluid (b). Temporal changes in released amount of Mn-por from the alginate matrix when immersed in simulated gastric (open circles), or intestinal fluid (filled circles) (c). This experiment employed the Mn-Ls/Alg whose initial concentration of the Mn-por in the alginate matrix was 1.0 mM. *n*=2. Plots and error bars represent mean value standard deviation, respectively.

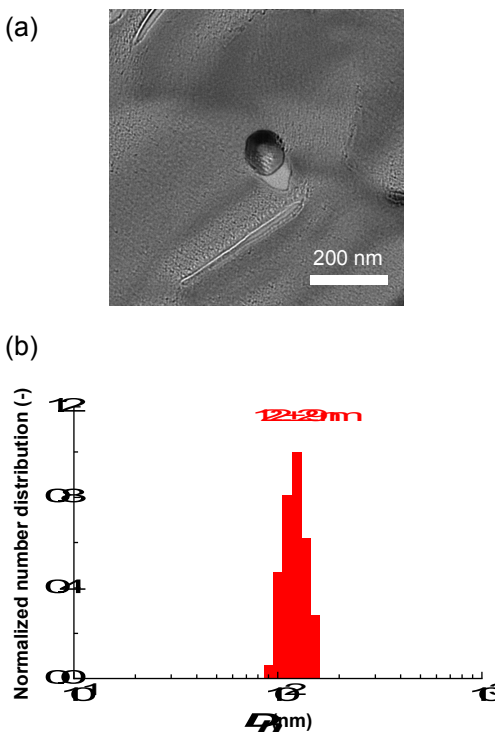

Figure 5. Confirmation of the integrity of Mn-Ls after exposure to simulated gastric and intestinal fluid. (a) FF-TEM image and (b) the size distribution, as determined by DLS of Mn-Ls after exposure. Values are expressed as mean ± SD. PDI = 0.06.

tract. Following treatment with GI fluids, the morphological integrity and $O_2{\cdot}^-$ inhibitory activity of the Mn-Ls was investigated using FF-TEM and cytochrome *c* assay, respectively. FF-TEM revealed that the Mn-Ls treated with the GI fluids retained their original structure (Fig. 5a), indicating that the Mn-Ls have a sufficient tolerance to gastric and intestinal fluids. The size distribution of the collected Mn-Ls was also comparable to their distribution prior to the digestion of the alginate matrix (Fig. 5b). $O_2{\cdot}^-$ inhibitory activity of the drugs was estimated by a conventional cytochrome *c* assay.[24] Mn-Ls after treatment with simulated GI fluids remained $O_2{\cdot}^-$ inhibitory activity. However, the levels of $O_2{\cdot}^-$ inhibitory activity relative to the control decreased from 42% to 31% by the treatment with GI fluids (Table 2). Although result of the assay indicated that treatment with the GI fluids slightly decreased $O_2{\cdot}^-$ inhibitory activity of the Mn-Ls, as discussed below, levels of *in vivo* efficacy on antitumor demonstrated that Mn-Ls exerted their therapeutic effect even after passing through the GI tract. Therefore, if $O_2{\cdot}^-$ inhibitory activity of drugs after GI treatment remains more or equal to present level (k_{obs}=1.8×10^{-3} s^{-1} at 5 μM), the drugs can exhibit *in vivo* antitumor effect to some extent.

Table 2. $O_2{\cdot}^-$ inhibitory activity of Mn-Ls

Samples	k_{obs} (s^{-1})	Relative inhibitory activity (%)**
Mn-Ls before GI treatment*	1.5×10^{-3}	42
Mn-Ls after GI treatment*	1.8×10^{-3}	31
Water (control)	2.6×10^{-3}	0

* In order to perform experiment within detection limit of the assay, concentration of Mn-por embedded in the liposomes was 5 μM.
** calculated by (1-[k_{obs} /$k_{obs\ (control)}$])×100.

Evaluation of *in vivo* antitumor efficacy of Mn-Ls/Alg

We determined the antitumor efficacy of orally administered Mn-por

by monitoring the size of tumors in mice transplanted with Colon-26 cells following oral administration of Mn-Ls/Alg. Compared to the control group, the treatment group had significantly smaller tumors 8 days after transplantation ($p < 0.01$) (Fig. 6a). This result clearly shows that oral administration of Mn-Ls/Alg effectively inhibited tumor growth. Even by day 8, the tumor sizes in the treatment group continued to remain small and were difficult to measure (Fig. 6b). A more detailed understanding of the biokinetics of Mn-por in the body is required in order to determine its therapeutic mechanism.

Discussion

There have been several reports about *in vivo* therapeutic efficacy of manganese porphyrins as an SOD mimic. Kawakami *et al* showed that intraperitoneal administration of manganese porphyrins could suppress cardiac dilation of SOD-deficient mice.[14] Furthermore, Kawakami *et al* demonstrated that portal vein injection of manganese porphyrin conjugated with catalase exhibited protective effect against hepatic ischemia/reperfusion injury *in vivo*.[16] Our group demonstrated that survival rate of paraquat-treated mice was improved by intraperitoneal injection of Mn-por incorporated in niosomes.[22] However, the *in vivo* therapeutic efficacy of orally administered SOD mimics has not been reported. In this study, we demonstrated the *in vivo* antioxidant and antitumor efficacy of Mn-por following its oral administration into oxidatively stressed mice. The therapeutic effects of orally administered Mn-por can be attributed to two reasons: (i) the alginate hydrogel fibers used as a carrier matrix were pH-sensitive; and (ii) both Mn-Ls and Mn-por remained intact following digestion of the carrier matrix.

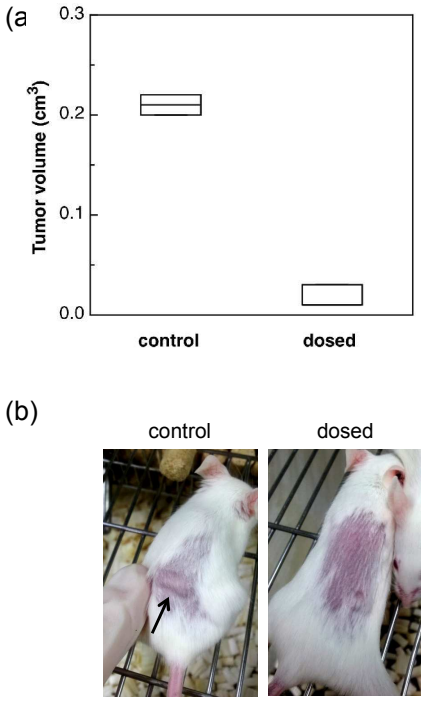

Figure 6. *In vivo* antitumor efficacy of orally administered Mn-Ls/Alg. (a) Comparison of tumor volume in mice ($n = 5$, $p < 0.01$) transplanted with Colon-26 cells. In the box plot, upper and lower lines of the box stand for upper and lower quartiles. The centered line represents the median. (b) Representative images of the region containing Colon-26 tumors. These data were obtained on day 8 post-transplantation. The location of the tumor is indicated by an arrow.

Effective oral administration of drugs normally requires encapsulation of the drugs in a carrier matrix. In the case of drug delivery to the intestine via oral route, the carrier matrix should be insoluble in the gastric fluid to retain the drugs within the matrix, and conversely, be soluble in intestinal fluid to allow the release of the drugs in the intestine. The alginate hydrogel fibers presented in this study exhibited ideal solubilization and drug release behavior in both of the simulated GI fluids (Fig. 4).

Mn-Ls could maintain its original structure even after encapsulating them in the alginate matrix and the treatment by the GI fluids (Fig. 5) During process of encapsulating Mn-Ls in the alginate matrix and the treatment with the GI fluids, increment of concentration of electrolyte is the most possible cause of aggregation/fusion of liposomes, which results in destruction of the liposomal structure. In general, liposomes consist of phospholipids do not aggregate even when exposing them to aqueous solution including considerably high concentration of monovalent electrolyte. For example, liposomes composed of egg yolk phosphatidyl choline can stably disperse in aqueous solution containing 1 M of NaCl or KCl.[28] This stable dispersion may be attribute to the hydration force,[29] whose mechanism has long been controversial yet. Concentration of monovalent electrolytes used as simulated GI fluids was significantly lower than that can induce aggregation/fusion of liposomes. Sarmiento *et al* reported that aggregation/fusion of liposomes did not occurred when concentration of Ca^{2+} (divalent cation) was up to 0.7 M.[30] However, we used aqueous solution containing 1.8 M (20 wt%) of Ca^{2+} for encapsulating Mn-Ls in alginates matrix, which exceeded the critical concentration. Despite exceeding the critical concentration of Ca^{2+}, aggregation/fusion of Mn-Ls was not observed in this study. We assumed that the inhibition of aggregation/fusion of liposomes in high concentration of Ca^{2+} (1.8 M) might be attributed to the presence of alginate anions, which can cancel the charge of Ca^{2+}. However, the mechanism underlying this inhibition effect remained unknown.

In this study, we have demonstrated that orally administered Mn-por can efficiently suppress tumor growth (Fig. 6). This suppression may be attributed to the $O_2{\cdot}^-$ inhibitory activity of Mn-por. This speculation is supported by previous reports that have described the role of SOD in the reduction of tumor malignancy. For example, Oberley *et al* showed that malignant cell growth was inhibited by manganese superoxide dismutase (MnSOD) overexpression.[31] According to this report, accumulation of H_2O_2 derived from the catalytic reaction of MnSOD leads to suppression of tumor growth. We have also demonstrated that Mn-por can catalyze the conversion of superoxide to H_2O_2.[22] Thus, the mechanism of tumor suppression described in the present study may be the same as

that suggested by Oberley *et al.* A more detailed analysis of the mechanism underlying tumor growth inhibition is required. If the generated H_2O_2 can act as a tumor-suppressor, the levels of H_2O_2 and Mn-por should increase simultaneously in the tumor. The pharmacokinetics of the drugs also still remains unclear. We speculate that the Mn-Ls should be adsorbed from epithelial of the intestine, and then be transported to the tumor tissue through the blood stream. During the transport through the blood stream, the enhanced permeation and retention effect should serve the more effective uptake of the drugs. On the other hand, free Mn-por may be bound to alginate polymer electrostatically, resulting in limiting adsorption of the free Mn-por from the epithelial. As a result, the free Mn-por should be egested from the intestine. These aspects require further investigation.

Mn-Ls/Alg exhibited significant antitumor effect in *in vivo* testing. Nevertheless, the present drug formulation remained two matters: (i) low encapsulation efficiency of the drugs (Table 1); and (ii) slight reduction of the $O_2{\cdot}^-$ inhibitory activity after exposing GI environment (Table 2), which should be solved for further drug efficacy. Encapsulation efficiency of Mn-Ls in the alginate matrix would be improved by increasing concentration of sodium alginate solution. The use of sodium alginate with higher concentration would form dense alginate polymer networks, which result in better retention of Mn-Ls in the matrix. Moreover, the use of concentrated alginate solution would improve the $O_2{\cdot}^-$ inhibitory activity of the drug. When using concentrated alginate solution, influx of gastric fluid would be limited due to dense polymer network being turned to be hydrophobic in response to gastric environment. It can be expected that preventing influx of gastric fluid address the problem of reduction of $O_2{\cdot}^-$ inhibitory activity because influx of gastric fluid may be a factor in reducing the $O_2{\cdot}^-$ inhibitory activity of Mn-por.

Conclusion

In summary, we have designed a formulation appropriate for the oral delivery of Mn-por, which mimics superoxide dismutase. In this study, to protect the drug from the harsh environment of the GI tract, alginate hydrogel was used as the carrier matrix. The alginate matrix did not dissolve in the simulated gastric fluid and, indicating that the Mn-Ls encapsulated in the matrix can be successfully delivered to the intestine. Conversely, the matrix dissolved in the simulated intestinal fluid within a few minutes and released the Mn-Ls. Even after exposure to the GI fluids, the Mn-por retained the $O_2{\cdot}^-$ inhibitory activity to some extent, indicating that the Mn-por can exert its activity even in the GI tract environment. This finding shows that alginate is a suitable carrier matrix, which allows orally administered drugs to be delivered to the intestine. Furthermore, results from animal experiments show that oral administration of Mn-por encapsulated in the alginate matrix has antitumor effects. Although there still remains problem regarding encapsulation efficacy of Mn-Ls in the alginate matrix and slight reduction of the $O_2{\cdot}^-$ inhibitory activity by treatment of the GI fluids, the drug formulation presented in this study has great potential for use as an orally available tumor suppressor owing to the $O_2{\cdot}^-$ inhibitory activity of Mn-por.

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