Near-infra-red fluorescent chitosan oligosaccharide lactate for targeted cancer imaging and photothermal therapy

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ABSTRACT
Photothermal therapy (PTT) is a promising approach for effective cancer treatment because of its non-invasive procedure, low toxicity to normal tissues, and high tumour ablation efficiency. Developing a PTT agent with precise tumour imaging capabilities is an essential prerequisite for effective PTT. In this study, we developed a bifunctional near-infra-red (NIR) fluorescent conjugate consisting of chitosan oligosaccharide lactate (COL) and the ZW800-1 NIR fluorophore (COL-ZW). We demonstrate that this conjugate is easy to use and that it is an effective theranostic agent for fluorescence-guided photothermal treatment. The temperature of COL-ZW increased by 62.3 °C after NIR laser irradiation (1.1 W/cm²) for 5 min in HT-29 tumour-bearing mice. The HT-29 tumours targeted by COL-ZW showed a remarkable decrease in tumour volume until a week after photothermal treatment. These in vivo results demonstrate that the bifunctional COL-ZW generates strong fluorescence and light-triggered PTT in tumour sites, indicating successful fluorescence-guided PTT. Importantly, no tumour recurrence or treatment-induced toxicity was observed after a single dose of COL-ZW with laser irradiation. Therefore, a combinatorial treatment with COL-ZW and NIR laser irradiation could serve as a promising strategy for photothermal cancer therapy.

Introduction
To date, a number of technologies for cancer treatments have been developed including chemotherapy, radiotherapy, photodynamic therapy, photothermal therapy (PTT), and combination therapy. Among these options, PTT has great potential for effectively treating cancers because of its non-invasive procedure, low toxicity to normal tissues, and high tumour ablation efficiency [1–7]. Photothermal therapeutic agents that exhibit strong absorbance in the near-infra-red (NIR) region can convert light energy into heat energy to induce hyperthermia (>48 °C) and kill tumour cells [8–12]. Although various materials including inorganic and polymeric nanoparticles have been explored as photothermal agents, many such materials are associated with certain disadvantages that limit their use, such as non-biodegradability, insufficient photothermal conversion efficiency, and unsolved biosafety issues [13–16].

Chitosan oligosaccharide lactate (COL) is a natural, biodegradable, non-toxic, cationic carbohydrate polymer, which is the depolymerised product of chitosan with a lower degree of polymerisation (≈30) and an average molecular weight (MW ≈ 5000 Da). Because of its high water solubility, short chain length, low viscosity, and antitumor properties, COL is suitable for pharmaceutical and biomedical applications [17,18]. The potential of COL as a carrier for imaging agents and anticancer drugs has been suggested due to its efficient intracellular and site-specific delivery [19–24]. Due to its tumour targetability, the biocompatible COL could be successfully used for PTT when combined with NIR fluorophores to act as an imaging as well as photothermal agent.

ZW800-1, a zwitterionic NIR fluorophore, is a promising candidate for photothermal cancer therapy because it shows remarkable optical properties including a high molar extinction coefficient, relatively high quantum yield, and good photostability in warm serum [25,26]. Moreover, ZW800-1 itself exhibits no serum binding, has ultralow non-specific tissue uptake, and is rapidly excreted from the body. These properties are more desirable than those of the FDA-approved NIR dye indocyanine green (ICG) and other commercial dyes like Cy5.5 and IRDye800CW [25,27]. As it is very important to preserve the tumour targetability of COL after conjugation with an NIR fluorophore, ZW800-1 could serve as an optimal NIR photothermal agent based on its optical properties and in vivo performance.

In this study, we prepared an NIR fluorescent agent COL-ZW by conjugating the ZW800-1 NIR fluorophore to COL for tumour-targeted imaging and photothermal treatments in vivo. Bifunctional COL-ZW was capable of targeting the...
tumour tissue and allowed for the monitoring of time-
dependent accumulation of COL-ZW in the tumour, conse-
quently enabling the determination of the optimal timing for
photothermal treatment. We thus demonstrated that COL-ZW
may be a safe and reliable theranostic modality for fluo-
rescence-guided photothermal cancer therapy in clinical ap-
lications. To our knowledge, this is the first report of using COL
in combination with the ZW800-1 NIR fluorophore as a sim-
ple and effective method for targeted photothermal treat-
ment of cancer.

**Materials and methods**

**Materials**

All chemicals and solvents were of American Chemical
Society grade or HPLC purity. COL (average MW ≈ 5000 Da,
> 90% deacetylated form) was purchased from Sigma-Aldrich
(St. Louis, USA). ZW800-1 NHS ester was prepared as
a 90% deacetylated form) was purchased from Sigma-Aldrich.

**Conjugation of COL to ZW800-1 NIR fluorophore
(COL-ZW)**

COL (1 μmol, 5 mg) was conjugated to the ZW800-1 NHS
ester (1 μmol, 1 mg) in phosphate-buffered saline (PBS; 2 ml,
pH 8) at room temperature for 6 h. The reaction mixture was
purified using a gel-filtration chromatography (GFC) system
with Econo-Pac P6 cartridges (Bio-Rad, Hercules, USA) and a
flow rate of 1 ml/min (PBS, pH 7.4). Size distribution and
diameter of COL and COL-ZW in water were measured by
dynamic light scattering (DLS, Nano ZS Zetasizer, Malvern
Instruments Ltd., UK).

**Optical property measurement**

Optical measurements were carried out at 37 °C in PBS, pH
7.4. Absorption and fluorescence spectra of COL-ZW conju-
gate were detected using a fibre optic flame spectrophotom-
eter (Ocean Optics, Dunedin, USA). NIR excitation was
generated by 5 mW of 655 nm red laser pointer (Opcom Inc.,
Xiamen, China) connected with a 400 μm NA 0.22 fibre
(Ocean Optics) [28].

**In vitro cancer cell binding assay**

The human colorectal adenocarcinoma cell line, HT-29 and
the human breast adenocarcinoma cell lines, MCF-7 and
MDA-MB-231 were purchased from the American Type
Culture Collection (ATCC, Manassas, USA). The cancer cells
were cultured in Roswell Park Memorial Institute (RPMI) 1640
medium supplemented with 10% foetal bovine serum (FBS,
Gibco BRL, Paisley, UK) and an antibiotic-antimycotic solution
(100 units/mL penicillin, 100 μg/mL streptomycin, and
0.25 μg/mL amphotericin B; Welgene, Daegu, South Korea) in
a humidified 5% CO2 atmosphere at 37 °C. The final concen-
tration of 2 μM COL-ZW was added when the cells attained
50 ~ 60% confluence. After 1 h incubation at 37 °C, the cells
were washed with PBS and imaged using a Nikon Eclipse Ti-
U inverted microscope system (Nikon, Seoul, South Korea) [28].

**In vitro photothermal cytotoxicity**

Calcein-AM (green for live cells) and propidium iodide (red
for dead cells) fluorescent stains were used to visualise the
PTT-induced cell death in COL-ZW treated cells. Initially, HT-
29 cells (1 × 104 per well) were seeded into a 24-well plate.
After 24 h, the cells were treated with 2 μM COL-ZW for 1 h
and washed with PBS. Then, PTT was performed under
808 nm laser at 1.1 W/cm² for 5 min. After PTT, the cells were
allowed to incubate for another 3 h and then costained with
calcein-AM and propidium iodide for 30 min. After washing
twice with PBS, the stained cells were observed under a
fluorescent microscope (Nikon).

**HT-29 xenograft mouse model**

Animal experiments were carried out in accordance with pro-
tocols approved by Chonnam National University Animal
Research Committee (CNU IACUC-H-2017-64). Male NCRNU
nude mice (6 weeks old, ~25 g) were obtained from Orient
(Seongnam, South Korea). HT-29 cancer cells were harvested
in 100 μL PBS with 1 × 10⁶ cells per mouse and subcutane-
ously inoculated into the right flank of each mouse. The
COL-ZW conjugate was intravenously injected when tumours
attained a size of 1 cm in diameter. Animals were anaesthe-
tized and imaged over the entire time period [28].

**In vivo NIR fluorescence tumour imaging**

In vivo NIR fluorescence imaging was carried out using a
FOBi imaging system (NeoScience, Suwon, South Korea).
Tumour-to-background ratios (TBR) as fluorescence/back-
ground signals were analysed by ImageJ software. The back-
ground means the fluorescence signal of a region
neighbouring to the tumour during the imaging time period.
To validate in vivo antitumor effects, tumour volumes were
observed over the entire time period and determined by the
following formula: $V = 0.5 \times \text{longest diameter} \times \text{(shortest
diameter)}^2$ [28].

**Evaluation of in vivo photothermal effect**

HT-29 tumour-bearing mice were subjected to intravenous
injection of PBS or COL-ZW. After 4 h injection, mice were
anaesthetized and the tumour sites were exposed to the
laser irradiation (1.1 W/cm², $\lambda = 808 \text{nm}$) for 5 min. Tumour
temperature was confirmed using a FLIR™ thermal imager
(FLIR Systems,., Wilsonville, USA), and temperature changes in
the tumour sites were observed every 1 min from the initial
stage of the laser irradiation over the entire time period.
After 24 h irradiation, tumours were collected from the laser-
treated mice for histological examination using haematoxylin
and eosin (H&E) staining [28].
**Statistical analysis**

Statistical analysis was carried out using a one-way ANOVA followed by Tukey’s multiple comparisons test. Statistically significant differences were considered to be at a level of \( p < .05 \). Results are expressed as mean ± SD and curve fitting was carried out using the Prism software (GraphPad, San Diego, USA) [28].

**Histological examination**

Collected tumours were fixed in 2% paraformaldehyde and flash frozen in liquid nitrogen after embedding in optimal cutting temperature (OCT) compound. Frozen tumour tissues were cryosectioned with a 10 \( \mu \)m thickness followed by staining with H&E. Histological examination was carried out using a Nikon Eclipse Ti-U inverted microscope system (Nikon) [28].

**Results and discussion**

**Preparation and characterisation of the COL-ZW conjugate**

The procedure for COL-ZW synthesis is shown in Figure 1(a). The amine groups of COL were covalently conjugated to the ZW800-1 NHS ester through amide bond formation via a condensation reaction performed in PBS, pH 8 at room temperature for 6 h. To preserve the tumour targetability of COL, the conjugation reaction was initiated a molar ratio of 1:1 of COL to ZW800-1. The advantage of using the ZW800-1 NIR fluorophore is that there is no need to consider any potential changes to the properties of COL after the conjugation reaction because the charge-balanced ZW800-1 is optimised for labelling peptides, especially small molecules, as demonstrated previously [27]. The COL-ZW conjugate was purified in a GFC system as shown in Figure 1(b) and demonstrated successful conjugation and favourable characteristics for further in vitro and in vivo studies. In addition, the size distribution and diameter of COL and COL-ZW conjugate were examined by DLS measurements (Figure 1(c)). As expected, the COL was found with nanoaggregates in the size range with diameter 150–200 nm in water, because chitosan is known to form aggregation having a hydrophobic core and a hydrophilic surface in aqueous solutions. Interestingly, the size of COL-ZW conjugates was measured in the 1–1.5\( \mu \)m size range and much smaller than COL nanoaggregates. This indicates that a COL-ZW conjugate may not form the same assembly process that of the COL in water, because the charge-balanced ZW800-1 plays an important role by preventing the self-assembly of COL.

As the COL-ZW conjugate has high water solubility, the molar extinction coefficient and quantum yield \((\varepsilon = 2,46,000 \text{ M}^{-1}\text{cm}^{-1}, \Phi = 13.5\%\) are significantly higher than those of ICG \((\varepsilon = 111,060 \text{ M}^{-1}\text{cm}^{-1}, \Phi = 1.7\%\) in aqueous conditions [26,29]. Figure 1(d) shows the optical spectra of COL-ZW in PBS. COL-ZW showed an absorbance peak at 768 nm and yielded the highest fluorescence at 789 nm. Although the absorption peak of COL-ZW did not match the 808 nm laser diode used for photothermal treatments, the photothermal effect can still be triggered by the 808 nm laser diode due to the high molar extinction coefficient of the ZW800-1 NIR fluorophore.

**Assessment of in vitro photothermal effect**

For real-time photothermal imaging, a FLIR® thermal imager equipped with an 808 nm laser diode (0–2 W) was used to measure the photothermal conversion efficiency of the COL-ZW conjugate. The photothermal effect was confirmed in vitro by monitoring the following solutions: 100 \( \mu \)M COL-ZW prepared in PBS and PBS alone, during irradiation with
an 808 nm NIR laser (1.1 W/cm²) for 1 min. The concentration of COL-ZW used was equivalent to the 0.4 mg/kg single dose of ZW800-1 NIR fluorophore used previously for in vivo studies [26]. As the irradiation time of the COL-ZW solution increased, the colour of the photothermal images dramatically changed from dark purple (indicating low temperature) to bright yellow (indicating high temperature) within 1 min, compared to minimal colour change in PBS alone (Figure 2(a)). The temperature of the COL-ZW solution increased rapidly from ambient temperature (25.3 °C) to 87.7 °C, while no obvious change was observed for PBS alone at 1 min post-irradiation. These results demonstrated that the COL-ZW solution can absorb the 808 nm laser light and convert the absorbed energy into a considerable amount of thermal energy in a time-dependent manner. In terms of stability, the COL-ZW conjugate presents stable physicochemical and optical properties in PBS for one week, as proven by the constant absorbance values (Figure 2(b)). However, the photostability of COL-ZW solution gradually decreased during the 5 min of laser irradiation, which indicates the ZW800-1 NIR fluorophore degraded after showing the photothermal conversion performance (Figure 2(c)). To further evaluate the photothermal stability under repeated laser irradiation, three cycles of irradiations were performed as shown in Figure 2(d). As expected, the temperature of COL-ZW solutions rapidly elevated in the first cycle and significantly decreased in the second and third cycles under repeated laser irradiation. This indicates that the COL is unable to protect ZW800-1 from photodegradation. Furthermore, an important consideration is that the photothermal conversion efficiency in vivo is highly dependent on the tumour targetability of COL-ZW conjugate; which is unlike the case for in vitro photothermal effects.

**In vitro cancer cell binding**

To identify the binding affinity of the COL-ZW conjugate to cancer cells, 2 μM COL-ZW solution was incubated with either HT-29, MCF-7, or MDA-MB-231 human cancer cell lines for 1 h at 37 °C. COL-ZW conjugates localised to the cell boundaries with high fluorescence intensities in all three cancer cell lines (Figure 3(a)). These results confirm that the COL-ZW can adhere to cell membranes, thereby delivering thermal energy directly to the cell membrane. As ZW800-1 alone showed no cellular uptake under any conditions [27], COL may play a crucial role in targeting COL-ZW to cancer cells. The higher cellular uptake of COL-ZW conjugates is most likely due to the positive charge of COL, which allows the COL-ZW conjugate to adhere to the negatively charged outer surfaces of cell membranes.

In order to visually demonstrate the laser-induced cellular photothermal effect, HT-29 cells were costained with calcein-AM and propidium iodide to identify live and dead cells, respectively, after photothermal treatment under the 808 nm laser irradiation (1.1 W/cm²) for 5 min (Figure 3(b)). As expected, COL-ZW induced massive cell death and showed intense homogeneous red fluorescence from propidium iodide, while no green fluorescence from calcein-AM was detected after the laser irradiation. This result suggests that the COL-ZW under laser irradiation could generate thermal energy and induce cell death.
In vivo NIR fluorescence imaging for tumour targetability

Accumulation of the COL-ZW conjugate in the tumours of HT-29 tumour-bearing mice was monitored in real time at different time points after intravenous injection for 24 h (Figure 4(a)). NIR fluorescence intensities of the tumour tissue increased within 2 h, and then gradually declined by 24 h post-injection. Based on TBR values, the photothermal treatments were performed at 4 h post-injection to prevent damage to normal tissues in the vicinity of tumour tissues due to high fluorescence levels in the skin at earlier time points (Figure 4(b)). These observations indicate that the COL-ZW conjugate could be useful for targeted tumour imaging, and that it can provide fluorescence-based guidance in real-time for effective photothermal treatments.

As shown in Figure 4(c), tumours showed no uptake of ZW800-1 alone for up to 4 h after injection. This result corresponds to those from a previous study [3,30]. Additionally, we confirmed the biodistribution of the COL-ZW conjugate by imaging fluorescence in major organs resected from mice (Figure 4(d)). COL-ZW mainly shows rapid renal clearance at 4 h post-injection without significant uptake in other tissues. As the COL-ZW conjugate has high tumour targetability and low non-specific tissue uptake within a short period of time, COL-ZW could be useful for addressing unmet clinical needs.

Assessment of in vivo photothermal effect

Based on the high photothermal conversion efficiency of COL-ZW in vitro, the PTT capability of COL-ZW in vivo was
further studied using the HT-29 tumour-bearing mouse model. Importantly, the optimal power density of an 808 nm laser was investigated in a PBS-treated group to prevent the photothermal effect generated by only laser power without the photothermal agent. With the increase of the laser power from 1.1 W/cm² to 1.2 W/cm², temperatures in tumour sites continuously increased by \( \frac{\Delta T}{\Delta t} \) during the 5 min of laser irradiation, which is dependent to the laser power density (Figure 5(a)). Although a power density of 1.0 W/cm² is still available in this study, the power density of 1.1 W/cm² was optimally selected to maximise the PTT efficiency of COL-ZW.

Mice were intravenously injected with COL-ZW (100 µM based on the ZW800-1 NIR fluorophore) at 4 h before laser irradiation. Tumour sites were then irradiated with an 808 nm laser with a power density of 1.1 W/cm² for 5 min. As shown in Figure 5(b), the tumour temperature showed a rapid increase to \( \Delta T = 56^\circ C \) within 2 min after laser irradiation, and the photothermal treatment was maintained for an additional 3 min for effective tumour ablation. This demonstrates that the temperature change in tumours was generated by the strong photothermal effects of COL-ZW. Additionally, the real-time temperature variation at the tumour boundary was monitored using a FLIR™ thermal imager (Figure 5(c)). After laser irradiation, only a mild temperature increase to \( \Delta T = 41.2^\circ C \) was observed in case of tumours treated with PBS alone, while a rapid temperature increase to \( \Delta T = 62.3^\circ C \) was observed in tumours treated with COL-ZW. These results indicate that the increased tumour temperature is sufficient to kill cancer cells without damaging the adjacent normal tissue.

**In vivo PTT efficacy**

To further confirm the therapeutic effects of the COL-ZW conjugate, HT-29 tumour-bearing mice were carefully monitored for 5 days after photothermal treatment (Figure 6(a)). The tumour sizes in PBS- or COL-ZW-treated groups were measured every other day. Tumour volumes in the COL-ZW-injected group gradually decreased within 5 days after laser irradiation, and ultimately only the black scars on the initial tumour sites remained. In contrast, tumours in the PBS group markedly increased over time without any apparent effects of laser irradiation (Figure 6(b)). These results suggest that a
Figure 5. (a) Photothermal curves of PBS-injected mice at tumour sites irradiated with different power densities (1.0 W/cm², 1.1 W/cm², and 1.2 W/cm²) for 5 min. (b) Temperature changes in tumour sites in each treatment group were monitored during the 808 nm laser irradiation (1.1 W/cm²) for 5 min. Data points represent mean ± SD of three independent experiments. (c) Whole-body photothermal images of tumour-bearing mice at 4 h post-injections of PBS and COL-ZW, respectively, on irradiation with an 808 nm laser (1.1 W/cm²) for 5 min. Maximum tumour temperatures were automatically recorded using an infra-red thermal camera as a function of irradiation time.

Figure 6. (a) In vivo NIR photothermal therapeutic efficacy. Representative photos of tumour size changes after 808 nm laser irradiation (1.1 W/cm²) for 5 min at 4 h post-injection with PBS and COL-ZW. (b) Tumour growth rates in each treatment group were monitored for 7 days. Data points represent mean ± SD of three independent experiments. Tu: tumour. Scale bars = 1 cm. (c) Tumour H&E-stained slices of PBS and COL-ZW injected mice at 24 h after laser irradiation. Scale bars = 100 μm.
combination of COL-ZW injection and laser irradiation can suppress tumour growth. Moreover, no tumour recurrence or treatment-induced toxicity was observed in the COL-ZW group for 10 days after photothermal treatment. We therefore demonstrated that the COL-ZW conjugate has an excellent PTT efficacy from a single-dose treatment.

The tumours were resected at 24 h after laser irradiation, and examined using H&E staining for histological analysis. As expected, evidence of cell damage, including nuclear damage and cell shrinkage, were observed in tumours treated with COL-ZW and laser irradiation, while tumour tissues in the PBS and laser-treated group contained intact cells and exhibited normal patterns of cell proliferation without photothermal effects (Figure 6(c)). This result confirms the high hyperthermic therapeutic efficacy of COL-ZW in vivo and indicates that the COL-ZW conjugate is a biocompatible and effective PTT agent that can be safely used in cancer treatment.

Conclusions

Although many different types of PTT nanomaterials have been reported to solve the longstanding biosafety problems, the issues of uniformity, reproducibility and practical synthesis still remain for many PTT nanomaterials as they move from laboratories to clinical trials. In the present study, we showed that COL-ZW, consisting of natural COL and the biocompatible ZW800-1 NIR fluorophore, can be prepared by a simple one-step process without the requirement of organic solvents. Furthermore, tumour targeted COL-ZW was successfully utilised as a theranostic modality for fluorescence-guided photothermal treatment. COL-ZW showed strong absorbance in the NIR region, and exhibited tumour-specific targeting, high photothermal conversion efficiency, and effective tumour ablation. Photothermal tumour ablation could be induced by increasing the temperature of COL-ZW by triggering it with an 808 nm laser. COL-ZW is especially effective for photothermal cancer treatment due to the high tumour targetability of COL and the excellent photothermal property of the ZW800-1 NIR fluorophore. Therefore, COL-ZW is a promising potential PTT agent for enhancing the NIR fluorescence signal in tumour sites in order to provide more accurate guidance for photothermal cancer treatments. Overall, the biocompatible, bifunctional, COL-ZW conjugate has great potential for use in the development of next-generation cancer treatments.

Disclosure statement

The authors declare that there are no conflicts of interest. The authors are responsible for the conduction of experiments and writing of the paper.

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