A Novel NIR-II Theranostic Agent: A Win-win Strategy of Tracing and Blocking Tumor-associated Vessels for Oral Squamous Cell Carcinoma

Zheng Wei (✉ DG1935144@smail.nju.edu.cn)

Nanjing University Medical School Affiliated Stomatological Hospital: Nanjing Stomatological Hospital

Hongbo Zhang

Nanjing University Medical School Affiliated Stomatological Hospital: Nanjing Stomatological Hospital

Huihui Zou

Nanjing University Medical School Affiliated Stomatological Hospital: Nanjing Stomatological Hospital

Chuanhui Song

Nanjing Gulou Yi Yuan: Nanjing University Medical School Affiliated Nanjing Drum Tower Hospital

Sufeng Zhao

Nanjing University Medical School Affiliated Stomatological Hospital: Nanjing Stomatological Hospital

Zichen Zhao

Nanjing University Medical School Affiliated Stomatological Hospital: Nanjing Stomatological Hospital

Xinyu Zhang

Nanjing University Medical School Affiliated Stomatological Hospital: Nanjing Stomatological Hospital

Guorong Zhang

Nanjing University Medical School Affiliated Stomatological Hospital: Nanjing Stomatological Hospital

Yu Cai

Zhejiang Provincial People's Hospital

Wei Han

Nanjing University Medical School Affiliated Stomatological Hospital: Nanjing Stomatological Hospital

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Abstract

Tumor vessel co-option is a crucial predictor for tumor invasiveness but is easy to ignore in tumor vascular targeting therapy. A high density of tumor vessel co-option located alongside the tumor frontier predicted a high tendency of tumor invasion and regional metastasis. Herein, a novel NIR-II nanoagent (CS NPs) was constructed with an organic COi8DFIC dye and sorafenib, which demonstrated high tissue penetration and low tissue autofluorescence, enabling imaging of vessel co-option and tumor micrometastasis. This nanotherapeutic agent displayed considerably improved quantum yield of fluorescence (0.89%), high ROS generation and fairly good biosafety *in vivo*. Compared with indocyanine green (ICG), CS NPs exhibited better photostability and photothermal conversion efficiency. By tracking tumor-associated vessels and real-time tumor imaging, CS NPs could open/stop vascular targeting therapy by laser on/off. The combination of vessel disruption and imaging-guided photothermal therapy/photodynamic therapy provided a win–win strategy for oral squamous cell carcinoma (OSCC).

Introduction

Oral squamous cell carcinoma (OSCC) accounts for approximately 3% of cancers worldwide and is ranked as the 8th most fatal cancer^{1,2}. To date, surgery remains the major treatment for OSCC patients. However, surgery alone will lead to substantial esthetic and functional damage, which will greatly traumatize the patients^{3,4}. Thus, it is vital to acquire an early and accurate diagnosis and improve the therapeutic outcome for OSCC patients. Solid tumor growth largely depends on blood vessels⁵, which is a profound approach for nutrient transport and cellular metabolism⁶. It is well known that a corresponding supply of the tumor vascular system will be required as the tumor grows larger than 2 mm³^{7,8}. Vessels promote tumor growth and are closely related to malignant biological behaviors such as tumor recurrence, metastasis, and drug resistance^{8,9}. Moreover, it has been demonstrated that patients with a high density of microvessels in OSCC have a higher degree of malignancy, a higher ratio of lymph node metastasis and a worse prognosis^{10–12}. Therefore, strategies to block tumor-associated vessels by targeting angiogenesis will bring considerable excitement in cancer therapies.

Notwithstanding, antiangiogenic therapy has not met researchers' expectations over the past two decades, which should be attributed to antiangiogenic agents, such as bevacizumab, nintedanib, and sorafenib¹³, suffering from drug resistance or off-target challenges^{13–15}. Recently, several studies have indicated that tumors can continue to progress by hijacking the pre-existing vasculature of nonmalignant tissues, which is called vessel co-option¹⁶. The presence of vessel co-option can be clarified, to some extent, as the barrier of anti-angiogenic therapies, including unaccountable drug resistance and uncontrollable tumor metastasis^{17,18}. Cancer vessel co-option is a nonangiogenic process in which tumor cells directly hire the pre-existing vessels from the surrounding nonmalignant tissue, where cancer cells will finally infiltrate or migrate^{16,19}. Recently, a body of reports has revealed that neglecting vessel co-option might be the vital reason for anti-angiogenesis failure^{17,18,20,21}. Studies have highlighted that increased microvascular invasion could be one of the most independent prognostic factors in predicting

survival for OSCC patients^{22, 23}. Consequently, strategies targeting cancer vessel co-option and angiogenesis (tumor-associated vessels) at the same time will provide a survival benefit for cancer patients. Thus, it is necessary and urgent to realize real-time monitoring and imaging of these tumor-associated vessels.

Booming development has been witnessed in the optical bioimaging field during the past few years. Obtaining tumor-associated vascular information requires vascular details with high spatial and temporal resolution and real-time monitoring images. To date, fluorescence imaging (FLI) in the second near-infrared (NIR-II) window (900-1700 nm) represents a potent tool to achieve direct visualization of complex biological structures and processes and offers high-resolution images with enhanced precision²⁴⁻²⁷. Compared with near-infrared window fluorescence (NIR, 700-900 nm), NIR-II fluorophores have received tremendous attention due to their outstanding ability to penetrate deep tissue, high signal-to-background ratio (SBR), diminished tissue autofluorescence and reduced tissue scattering^{26, 28-31}. Thus, considering the unique capabilities of deep penetration, anti-quenching and a high signal-to-noise ratio, NIR-II probes are expected to have pronounced strength in intravital imaging of tumor-associated vessels and tumor outlines³²⁻³⁴.

Herein, we report an A-D-A-type nanoprobe (Coi8DFIC-sorafenib nanoparticles, CS NPs) focusing on high-contrast tumor association vessel mapping and high efficacy therapy by blocking tumor-associated vasculatures and boosting photodynamic therapy (PDT) and photothermal therapy (PTT) (Scheme 1). Basically, CS NPs were synthesized via nano coprecipitation, representing a large Stokes shift (~100 nm) with absorption/emission peaks at 838/1050 nm and a high level of fluorescence quantum yield ($QY_{CS\ NPs} = 0.89\%$). Owing to the long wavelength, CS NPs exhibited deep penetration over 6 mm, which benefited from precise traces of tumor-associated vessels. Briefly, novel multifunctional NIR-II nanoagent CS NPs with high biocompatibility demonstrate an excellent mapping strategy along with blocking tumor-associated vessel therapy for the first time, which may provide a promising win-win perspective for combatting tumors by inhibiting tumor-associated vessels (tumor angiogenesis and vessel co-option).

Experimental Section

Materials and agents

Coi

8DFIC ($C_{94}H_{76}F_4N_4O_4S_6$), sorafenib ($C_{21}H_{16}ClF_3N_4O_3$) and DSPE-PEG₂₀₀₀ were purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd. Indocyanine green (ICG, modified), 2,7 1,3-diphenylisobenzofuran (DPBF), 3,7-bis(dimethylamino)phenothiazine-5-yl chloride (methylene blue, MB) and tetrahydrofuran (THF) were purchased from Adamas-beta®. IR26 was obtained from Dezhou Longwei Pharmaceutical Technology Co., Ltd. (China). DSPE-PEG₂₀₀₀-rhodamine B was purchased from ToYong (Shanghai) Bio Co. Ltd. Deionized water was produced via a Millipore ultrapure water system (MUL9000 (A2)-H, China).

Fetal bovine serum (FBS) and Dulbecco's modified essential medium (DMEM) were purchased from Biological Industries (BIOIND, ISRAEL). Phosphate-buffered saline (PBS, 1×, pH = 7.4), penicillin/streptomycin (10,000 units/mL, 10,000 µg/ml, 100×) and Singlet Oxygen Green (SOSG) were purchased from Thermo Fisher Scientific. The Reactive Oxygen Species Assay Kit was purchased from Beyotime Biotechnology, China. Cell Counting Kit-8 (CCK-8) was obtained from Selleck Chemicals (Houston, TX, USA). LysoTracker Green DND-26, MitoTracker Deep Red FM and ER-Tracker™ Green were obtained from Nanjing Warbio Biotechnology Co., Ltd.

Characterization

A UV–vis-NIR spectrophotometer (Lambda 365) was employed for the absorption spectrum. Fluorescence spectrophotometry was carried out on a Hitachi F-7000 fluorescence spectrophotometer. NIR-II fluorescence was detected by NIR-II spectroscopy (Fluorolog 3) equipped with an InGaAs NIR detector with 808 nm laser irradiation. The hydrodynamic diameter was measured by a dynamic light scattering (DLS) system (DLS, BT-90). The morphology of the nanoparticles was detected by a Hitachi HT7700 transmission electron microscope (TEM, Japan). Fluorescence images were captured by a NIKON Ti (Japan) confocal microscope. Cellular flow cytometry was conducted on a FACSCalibur. The concentration of sorafenib was analyzed on a Q TRAP™ 5500 MS/MS system (Applied Biosystems, CA, US) coupled to a UFLC system (Shimadzu, Kyoto, Japan).

Preparation Of Cs Nps

In a typical procedure, CS NPs were prepared by nanoprecipitation. The mixture of COi8DFIC (1 mg) and sorafenib (1 mg) in THF (1 mL) was ultrasonically vibrated for 10 min at room temperature (RT). Subsequently, the COi8DFIC and sorafenib mixture was added to 10 mL deionized water containing 2 mg DSPE-PEG₂₀₀₀ via a microinjector with stirring. Then, THF was dismissed by vacuum rotary evaporation, and the final product was stored at 4°C. The CS NPs were tested at ratios of 1:1, 1:2, and 2:1 between COi8DFIC and sorafenib, and the most ideal ratio was selected for subsequent experiments. C NPs were prepared by the same method. To trace nanoparticles by NIR I fluorescence, C NPs and CS NPs were signaled with rhodamine B, which were named C-RB NPs and CS-RB NPs, respectively.

Embedding Rate and Drug Release

The concentration of sorafenib in CS NPs was quantified by UFLC-QTRAP-MS/MS. The concentration of COi8DFIC was examined via UV–Vis-NIR. The embedding ratio was calculated by the following formula:

$$\text{Embedding Ratio} = \frac{\text{Final concentration}}{\text{Original concentration}} \times 100\%$$

Sorafenib release behavior from CS NPs was detected in 3 pH environments (7.4, 6.5 and 4.5). Five milliliters of CS NPs was transfused into a dialysis bag (molecular weight = 1000 Da). Then, the dialysis bags were immersed in 500 mL of PBS at different pH values at room temperature with stirring. One hundred microliters of outer dialysate was collected at each detected time point. This releasing experiment was studied for 48 hours. Additionally, to simulate PTT-stimulated drug release in tumor tissue, we studied drug release at room temperature, 45 °C and 50 °C at a pH value of 6.5.

Stability Assessment

Aqueous solutions, PBS, DMEM and FBS solutions of CS NPs were placed at RT for 30 days at a concentration of 50 µg/mL (determined by sorafenib). Then, the hydrodynamic diameter was assessed on days 0, 7, 14 and 30.

Nir-ii Fluorescence Quantum Yield (Plqy) Detection

The organic dye IR26 ($\Phi_{\text{IR26}} = 0.05\%$ ^{35, 36}) was selected as a reference to calculate the quantum yields of C NPs and CS NPs. Five various concentrations were studied, and the 1000-1400 nm region fluorescence was plotted under an 808 nm laser. the PLQY was quantified followed the manner³⁷:

$$\Phi_{\text{sample}} = \Phi_{\text{IR26}} \frac{\text{Slope}_{\text{sample}}}{\text{Slope}_{\text{IR26}}} \left(\frac{n_{\text{sample}}}{n_{\text{IR26}}} \right)^2$$

$\Phi_{\text{IR26}} = 0.05\%$. The slope is the linear fitting line with the absorption as the X axis and the fluorescence intensity as the Y axis. n_{sample} and n_{IR26} are the refractive indices of water and dichloroethane (DCE), respectively.

Ros Detection

DPBF was utilized as the ROS probe of CS NPs under 808 nm laser irradiation. In this study, different concentrations of CS NPs in deionized water (2.97 mL, 20 µg/mL) were mixed with DPBF (30 µL, 10 mM) in a quartz cuvette. Subsequently, the mixture was excited under an 808 nm laser with a power of 0.5 W for a total of 10 s, in which the UV absorbance of DPBF at 415 nm was read at each set-time point.

To calculate the quantum yield of ROS produced by Coi8DFIC, MB was used as the reference dye. In this study, Coi8DFIC and MB were dissolved in dichloromethane (DCM). In addition, the procedure was the same as upper one. The quantum yield of ROS was calculated using the following formula:

$$QY_{\text{sample}} = QY_{\text{MB}} \left(\frac{\text{Slope}_{\text{sample}}}{\text{Slope}_{\text{MB}}} \right) \left(\frac{F_{\text{MB}}}{F_{\text{sample}}} \right)$$

$$F = 1 - 10^{-\text{OD}}$$

Φ represents quantum yield of ROS; the slope is the linear fitting with the irradiation time as X axis and the UV - Vis absorption of DPBF at 410 nm after irradiation as Y axis; F represents the absorption correction factor of the sample and MB. The OD value is the UV–Vis absorption of the sample and MB at 665 nm.

Singlet Oxygen($^1\text{O}_2$) Detection

SOSG was employed to catch the $^1\text{O}_2$ produced by CS NPs. SOSG (50 μM) was mixed with CS NPs (20 $\mu\text{g/mL}$) or ICG (20 $\mu\text{g/mL}$), which were irradiated with an 808 nm laser at a power of 0.1 W/cm^2 for total 10 s. Then, the fluorescence intensity at 525 nm was recorded at each set-time point.

The $^1\text{O}_2$ QY of CS NPs was appraised based on the ICG using the following formula:

$$QY_{\text{Sample}} = QY_{\text{ICG}} \left(\frac{\text{Slope}_{\text{Sample}}}{\text{Slope}_{\text{ICG}}} \right) \left(\frac{F_{\text{ICG}}}{F_{\text{Sample}}} \right)$$

$$F = 1 - 10^{-\text{OD}}$$

The slope is the linear fitting line with the irradiation time as the X axis and the fluorescence intensity of SOSG at 525 nm after irradiation as the Y axis. The OD value is the UV–Vis absorption of ICG and CS NPs at 808 nm.

Photothermal Effect And Photostability

To evaluate the photothermal capability, C NPs and CS NPs in aqueous solutions at different concentrations (0, 10, 20, 40, 80 $\mu\text{g/mL}$) were irradiated with an 808 nm laser. Then, a study on the photothermal effect of laser power (0.5, 1, 1.5 and 2 W/cm^2) was conducted. During the experiment, temperature was recorded via a near infrared thermal camera (FLIR, Arlington, VA, E50). The photostability of Coi8DFIC and CS NPs was assessed by taking pictures and reading the absorption spectra of their solutions before and after laser irradiation, respectively. Moreover, the same procedure for the photostability of ICG was also conducted.

The photothermal conversion efficiency (η) was determined according to the following equations³⁸:

$$\eta = \frac{hS\Delta T_{\text{Max}} - Q_{\text{Dis}}}{I(1 - 10^{-OD})}$$

h : the heat transfer coefficient;

S : the bottom surface area of container, $S = 1 \text{ cm}^2$;

I : the power of the laser, $I = 1 \text{ W/cm}^2$

Q_{Dis} : the energy taken in by the same solvent without nanoparticles in the quartz cuvette after the same laser irradiation; In this experiment, the solvent is water. $Q_{\text{Water}} = 14 \text{ mW}$.

$$\Delta T_{\text{Max}} = \Delta T_{\text{max}} - \Delta T_{\text{surrounding}}$$

$$hS = \frac{\sum m_i C_i}{\tau_s}$$

$$\tau_s = \frac{t}{-\ln \theta}$$

$$\theta = \frac{T - T_{\text{sur}}}{\Delta T_{\text{Max}}}$$

Therefore,

$$\tau_s = \frac{t}{-\ln \theta} = \frac{\sum m_i C_i}{hS}$$

$$t = - \frac{\sum m_i C}{hS} \ln \theta$$

m : the mass of solvent and solute; $m_{\text{Water}} = 10^{-3}$; $m_{\text{CSNPs}} = 10^{-5}$;

C : the specific heat capacity of solvent and solute, $C_{\text{Water}} = 4.2 \times 10^3 \text{ J/g}$.

Ordinarily, the specific heat of water was much higher than that of other materials. Thus, the m and C of CS NPs were too small to neglect compared with the m and C of water.

Consequently,

$$t = - \frac{m_{\text{water}} C_{\text{water}}}{hS} \ln \theta$$

In fact, t represents the time that it takes from temperature cooling to room temperature.

Cell Incubations

HACAT cells, HUVECs and CAL 27 cells were cultured with complete medium in a 37°C, 5% CO₂ humidified chamber. The complete medium consisted of DMEM, 10% FBS and 1% penicillin/streptomycin.

Cellular Uptake

To detect cellular uptake, HUVECs and CAL 27 cells were incubated with 5 µg/mL CS NPs-RB. Time gradients were set to clarify the time point for maximum uptake, which could be represented by the maximum RB fluorescence in cells. Subsequently, the intracellular localization of CS NPs was investigated using LysoTracker and MitoTracker. Method was followed the instructions. The colocalization was calculated by ImageJ with Pearson's correlation coefficient.

Intracellular Ros Assessment

To assess total ROS generation, CAL 27 and HUVECs were cultured with 10 µg/mL CS NPs at the maximum uptake time point and then treated with 10 µM DCFH-DA. Cells were treated with 808 nm irradiation (100 W/cm²) for 10 min. ROS generation was analyzed by confocal microscopy.

Cytotoxicity in vitro

Cell proliferation curves and clone forming approaches were adopted to investigate the cytotoxicity of C NPs and CS NPs. To detect the proliferation ability, CAL 27, HUVECs and HACAT cells were seeded in 96-well plates and precultured overnight, and then the medium was replaced by C NP and CS NP concentrations equivalent to 0.94, 1.88, 3.75, 7.5, 15, 30 and 60.0 µg/mL. Thereafter, the cells were cultured for 24 h. The cell viability was assessed by CCK8. In addition, a growth curve was generated with a similar method.

For clone formation observation, CAL 27 and HUVEC single cell suspensions were seeded into 6-well plates at a density of 2000 cells per well. Then, the cell clones, which were stained with crystal violet

(0.5% w/v), were counted after a 2-week incubation.

Angiogenesis Assay

HUVECs formed tube-like structures on Matrigel (Corning, USA) with the introduction of vascular endothelial growth factor (VEGF 100 ng/mL). The same method was adopted with the previous study. After 12-16 h of incubation at 37°C, tubes could be observed. The medium was removed and subsequently changed to contain sorafenib, C NPs, and CS NPs at a concentration of 10 µg/mL. As a result, the tube-like structures broke up 4 hours after incubation. The results were captured with the microscope.

Cell Apoptosis Investigation

CAL 27 and HUVECs were seeded into 6-well plates overnight. The medium was replaced with fresh DMEM containing sorafenib, C NPs and CS NPs. After incubation for 12 h, cells in the C NP and CS NP groups were irradiated with an 808 nm laser (100 mW/cm²) for 10 min. Next, the cells were labeled by using an Annexin V-FITC/PI kit. Cells were analyzed by flow cytometry.

Hemolysis Assay

The hemolysis assay was performed to clarify whether the CS NPs were safe for intravenous administration. Fresh blood (1 mL) was collected from healthy mice and then centrifuged (1000 g, 10 min, 4 °C). Red blood cells (RBCs) were washed with PBS 3 times. In this study, 200 µL RBCs were treated with DI water, PBS, and different concentrations of CS NPs, in which PBS and DI water were employed as the negative and positive controls, respectively. Then, the absorbance of the hemoglobin released in the supernatant was monitored by a UV spectrophotometer at 570 nm.

Tumor Animal Model Establishment

Female BALB/c nude mice (4-5 weeks old, ~15 g) were purchased from Jiangsu Jicui Yaokang Biotechnology Co., Ltd. Anesthesia mice were subcutaneously injected with CAL 27 cells (1×10^6) on the insulation blanket. When the tumor volume reached ~100 mm³, mice were randomly assigned for subsequent experiments. The tumor volume was calculated using the formula

Volume = $\frac{\text{Length} \times \text{Width}^2}{2}$. All animal experiments were approved by the Animal Ethical and Welfare Committee of Nanjing University (IACUC—D2102071).

Nir-ii Fluorescence Imaging Of Microcapillary Tubes And Vessels

To evaluate the NIR-II fluorescence penetration depth, 5 µg/mL CS NPs were put into a capillary tube with a diameter of 1 mm. Then, they were placed under fresh chicken breasts with different thicknesses of 2 mm, 4 mm, 6 mm, 8 mm and 10 mm. The fluorescence intensity of these capillary tubes showed the penetration ability of CS NPs.

CAL 27 tumor-bearing mice were injected with 100 µL CS NPs under anesthetization by isoflurane. NIR-II fluorescence of CS NPs was first evaluated for its capability to track microvessels.

The full width at half-maximum (FWHM) of capillary tubes (0.5 mm) was assessed to evaluate the spatial resolution. The gray values of all NIR-II images were normalized by ImageJ-win64. Then, gray value statics were fitted with Gauss fitting to estimate FWHM via Origin software. Then, we further performed bioimaging of the hindlimb and its vessels. The signal-to-background ratio (SBR) was calculated using the following equation³⁹:

$$\text{SBR} = \frac{I_{\text{signal}}}{I_{\text{background}}}$$

Tumor And Vessel Co-option Imaging

To clarify the ability to trace tumor and tumor-associated vessels, mice were injected intravenously with 100 µL CS NPs (concentration of COi8DFIC equivalent to 50 µg/mL). Tumor-bearing mice were observed with the NIR-II fluorescence animal imaging system at designated time points. In the meanwhile, ICG was employed as a control. Finally, the biodistribution of NPs in the different parts of organs was analyzed.

Antitumor therapy in vivo

A total of twenty-five CAL 27 tumor-bearing mice were randomly divided into five groups (i. physiological saline, ii. Sorafenib, iii. CS NPs, iv. C NPs + 808 nm laser, v. CS NPs + 808 nm laser). Each mouse received five therapies at intervals of 5 days, and 100 µL of solution was injected intravenously with COi8DFIC or sorafenib at a concentration of 50 µg/mL. The 808 nm laser at the power of 0.5 W/cm² was performed 2 h after injection, and the irradiation time was 10 min. Additionally, the temperature at the therapy site was recorded. Before every treatment, the weight and tumor size of each mouse were documented. Upon finishing treatment, the mice were sacrificed by cervical dislocation for necropsy, and tumors and their major organs were collected for histopathological analysis. The tumor tissues were evaluated with H&E, CD 31 and TUNEL staining. Major organs were stained with H&E. All histopathological analysis procedures strictly followed the instructions.

Investigation of the immune effect of tumor-associated vascular blocking therapy

C3H mice were subcutaneously injected on the right back with SCC7 cells (1×10^6) on the insulation blanket under anesthesia. When the tumor reached 100 mm^3 , tumor- and tumor-associated vessels were located by NIR-II fluorescence. Then, PDT therapies were conducted with the guidance of precise orientations, especially for vessel co-options around the tumors. The concentrations of drugs and laser power were the same as the treatment for BALB/C nude mice. After 5 treatments, 1×10^6 SCC7 cells were injected at the opposite site (the left back) of the mice. Then, the left tumor size and immune cells in the blood were investigated. Immune cells, including CD3^+ , $\text{CD8}^+ \text{CD3}^+$, and CD4^+ T cells, were analyzed by flow cytometry.

Blood Circulation Analysis And Biosafety Assessment

Sprague–Dawley (SD) rats (female) were employed to assess the medical effect of NPs. Approximately 130 g SD rats were randomly divided into three groups (i. saline, ii. Sorafenib, iii. CS NPs). Each group contained three rats. For blood circulation, the rats were treated by gavage with various solutions (200 μL , at a dose of 2.5 mg/kg). Blood was taken from the tail artery and stored in heparinized tubes at each time point (5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 48 h). The concentration of sorafenib in blood was estimated by UFLC-QTRAP-MS/MS based on the calibration curve of sorafenib. To evaluate the biosafety of NPs, ~ 120 g SD rats were randomly divided into four groups (i. saline, ii. Sorafenib, iii. C NPs, iv. CS NPs). The rats were treated by gavage with various solutions (200 μL , at a dose of 2.5 mg/kg).

Then, we developed a sensitive liquid UFLC-QTRAP-MS/MS assay for the quantitative determination of sorafenib in rat plasma via vein injection at a dose of 10 mg/kg. All blood samples were extracted into anticoagulant tubes from rats fasting and skipping their morning dose of sorafenib. To calculate the pharmacokinetics of sorafenib, blood samples were obtained at four timepoints: prior to sorafenib injection and 0.25, 0.5, 1, 4, 8, 24, 48 and 50 h after the injection.

The half-life of the drug was calculated as follows:

$$K = (\ln C_0 - \ln C) / t$$

$$t_{\frac{1}{2}} = 0.693 / K$$

Clinic Resources

Patients

Patients were all collected from the Department of Oral and Maxillofacial Surgery, Nanjing Stomatological Hospital Affiliated Medical School of Nanjing University from 2013 to 2016. A total of 40 patients were enrolled in this study, and all patients were diagnosed with OSCC by the Department of Pathology. Data of all patients on age, sex, diagnosis lesion site, worst pattern of invasion (WPOI), survival, etc., were collected for subsequent analysis. Additionally, patient samples and relative statistics were all approved by the patients and the Research Ethics Committee of Nanjing Stomatological Hospital (NJSB-2021NL-019).

Pathological Study

All pathological progression was assessed by two individual experienced pathologists. All pathological assessments were based on the latest edition of the Classification guidance. WPOI types were assessed on H&E-stained sections. Tumor-associated vessels were confirmed by CD 31 immunohistochemical staining.

The vessel co-option located at the interface of OSCC and adjacent nonmalignant tissue was also marked with CD 31. Microvessel density (MVD) was employed to evaluate vessel co-option⁴⁰.

Surrounding the edge of OSCC, three zones (1.5 mm²) were randomly circled, and vessel co-option was counted in those circles. The MVD of vessel co-option was calculated using the following formula:

$$\text{MVD}(/ \text{mm}^2) = \frac{\text{SUM}}{1.5}.$$

Statistical analysis

The chi-square test and Fisher's exact test were used to estimate correlations among the vessel co-option mode, WPOI and survival. GraphPad, Origin, ImageJ and FlowJo were employed to analyze all data. For all statistical analyses, $p < 0.05$ was considered significant.

Results

Tumor associated vessels in OSCC

It is widely known that cancer cells have close interactions with vascular systems. In particular, a large number of cancers rely on the blood supply to progress. Recently, a body of reports has indicated that the expansion of tumors depends not only on angiogenesis inside it but also on the pre-existing vascular system in nonmalignant tissues, which are termed vessel co-options. Tumor angiogenesis and vessel co-option could be generated as tumor-associated vessels (Figure 1B, 1C).

Based on the report, there were two ways that blood vessels might be coopted: 1. Cancer cells directly replace normal epithelial cells; 2. Malignant cells invade the nonmalignant zone and employ pre-existing vessels¹⁸.

As shown in Figure 1, tumor angiogenesis and vessel co-options (indicated by the red arrow) existed in OSCC patient samples (Figure 1E, 1F). However, numerous studies have indicated that targeting angiogenesis shows only modest efficacy in patients and does not meet the high expectation due to low efficacy or drug resistance. Therefore, preexisting vessels drew our attention and were considered another major driver of tumor progression.

Vessel Co-option And Tumor Infiltration Patterns

Patterns of invasion indicate OSCC tumor invasiveness. In this study, we found that vessel co-option showed a close correlation with the invasive pattern. The worst pattern of invasion (WPOI) was assessed on H&E-stained slides. Both the CD 31⁺ zone and the zone located in the frontier of the tumor represented vessel co-options, as demonstrated in Figure 2A. A total of 40 patients were enrolled in this study, in which 5 types of WPOIs were included: 8 were type 1, 7 were type 2, 8 were type 3, 20 were type 4 and 7 were type 5. All data of 50 patients are shown in Table 1. The vessel co-options in all 5 types of WPOIs are shown in Figure 2B. Then, the relevance between the MVD of vessel co-option and WPOI was calculated (Figure 2C, Supplementary Table 2), and there was a comparable linear relationship between them. The higher MVD, the higher level of WPOI. The results proved that the MVD of vessel co-option could perfectly predict the mode of WPOI, and vessel co-option might be an effective biomarker to predict the invasiveness of OSCC. With the same method, there was a relevant relationship between MVD and T stage (Supplementary Table 3). Therefore, vessel co-option could be labeled with tumor biological behavior, and insights into vessel co-option would bring new expectations for OSCC therapy. Additionally, a growing body of literature suggests that tumors employing vessel co-option are more resistant to antiangiogenic therapies. Thus, strategies targeting tumor-associated vasculatures should include vessel co-option and angiogenesis inside tumors. Therefore, a novel win-win NIR-II nanoagent was designed to track the tumor-associated vasculatures in real time and target them by employing chemical blocking, PTT and PDT.

Synthesis And Characterization

An A-D-A-type π -conjugated small molecule, COi8DFIC, was demonstrated to be an excellent phototheranostic material. Herein, COi8DFIC and sorafenib, a vascular blocking drug, were combined by nano coprecipitation to produce nanoparticles named CS NPs (Figure S2), of which the embedding rate of sorafenib was $87.34 \pm 0.83\%$ and that of COi8DFIC was $80.28 \pm 3.53\%$. In this study, we proved that the best ratio of COi8DFIC and sorafenib was 1:1, while the characteristics of ratios 1:2 and 2:1 were not as good (Figure S2). Therefore, a ratio of 1:1 was employed for the subsequent experiments. With the same method, COi8DFIC NPs (C NPs) were also synthesized. From organic COi8DFIC solution to C NP hydrophilic solution, the maximal absorption displayed a large redshift of ~ 100 nm (Figure 3A). Then, the absorption spectra of CS NPs proved the success of the combination (Figure 3B). In addition to the longwavelength absorption, CS NPs also possessed several attractive characteristics. First, CS NPs

showed better photostability than ICG and COi8DFIC molecules (Figure 3C and Figure S2B). After 10 min irradiation at 808 nm (0.5 W/cm^2), the absorption spectrum of CS NPs exhibited no change, but ICG showed considerable decay. Next, characterized by TEM, the C NPs and CS NPs were $\sim 100 \text{ nm}$ and $\sim 120 \text{ nm}$, which was consistent with the DLS results of $\sim 90 \text{ nm}$ and 120 nm . (Figure 3D, 3E). The fabricated CS NPs displayed long-term stability in simulated physiological environments (PBS, DMEM and FBS, Figure 3F) at 37°C for 30 days (Figure S3).

Given the A-D-A structure, the aggregation property of COi8DFIC in NPs was activated, which showed a longwavelength redshift of 100 nm , reaching the NIR-II region (Figure 3G). π conjugated compounds are well known for their high performance in strong luminescence, of which the electron-donating “D” core delivers intense emission properties. Therefore, C NPs and CS NPs showed longwavelength NIR-II fluorescence (1052 nm) and high quantum yields (PLQYs) of 1.19% (23.82-fold of IR26) and 0.89% (17.85-fold of IR26), respectively (Figure 3H). The fluorescence of CS was not significantly quenched after 7 days of nondark storage at RT. (Figure 3I).

To further investigate the characteristics, the photothermal effect of CS NPs under 808 nm laser irradiation was examined. As shown in Figure 4A, the temperature rise depended on the concentration of CS NPs and the power of the 808 nm laser. The temperature of the CS NP solution ($20 \mu\text{g/mL}$) could rise from 24°C to 63°C upon 8 min of 808 nm laser (1 W/cm^2) irradiation (Figure 4A). As displayed in Figure S4A, there was no significant change in temperature after 3 heating-cooling cycles performed on CS NPs, indicating eminent photostability. The photothermal effect of C NPs was also studied and is shown in Figure S4B and 4C. Following the formula, the photothermal conversion efficiency of CS NPs was 46.94% (Figure 4A). With a high photothermal conversion efficiency and favorable stability, CS NPs are an excellent candidate for photothermal therapy.

Then, *in vitro* ROS generation of C NPs and CS NPs was evaluated by DFBP and SOSG probes, where MB and ICG were employed as the reference, respectively. As shown in Figure 4B, in the presence of MB and COi8DFIC, the absorption at 450 nm decreased, which meant that the generated ROS degraded DPBF. Based on the formula in the method, the total ROS quantum yield of COi8DFIC was 18.85 (33.07-fold of MB). Then, the SOS efficiency of CS NPs was evaluated with SOSG, which was determined by comparison with the commercial photosensitizer ICG. Under 808 nm laser irradiation, CS NPs induced decomposition of SOSG, which was shown as a reduction in the fluorescence intensity at 525 nm . The SOS quantum yield of CS NPs could be calculated by the reference of ICG (0.2%). The results revealed that CS NPs exhibited an ROS quantum yield of 0.92% , which was 4.60-fold that of ICG (Figure 4C). Compared with ICG, CS NPs possessed a higher capability of producing SOS and resistance to photobleaching, which made CS NPs a capable and durable PDT agent.

Cellular Uptake And Ros Generation

Given the above results, it was indicated that CS NPs performed well both in efficient NIR-II region fluorescence and laser-irradiated PDT/PTT effects. These characteristics make it a potential candidate for antitumor applications, including OSCC treatment. In addition, it was revealed that both vessel co-option and tumor angiogenesis inside the tumor itself played a significant role in patient prognosis. Thus, both of them should be regarded as the focus of tumor vascular occlusion therapy. According to previous results, it was demonstrated that the combination of sorafenib with PDT did a remarkable job in OSCC treatment⁴¹. Herein, the NIR-II photosensitizers COi8DFIC and sorafenib were combined to realize a win-win strategy in tracking tumor-associated vessels with deep penetration and high resolution and efficiently blocking tumor vessels.

The release of Sorafenib *in vitro* was studied. With laser irradiation time, smaller particles were present (Figure 5A), which could be due to the release of sorafenib. The release manner was first evaluated in different pH environments (pH = 7.4, 6.5, 5.3). Low pH could trigger more sorafenib released. Since the temperature would rise to 40 °C-50 °C when CS NPs (20 µg/mL) were irradiated with 0.3 W/cm² laser. The release of sorafenib at different surrounding temperatures was then studied. Compared with RT, 5.5-fold sorafenib could be delivered at 50 °C (Figure 5C). In addition, drug release increased with increasing acidity and temperature (Figure 5B and 5C). The results indicated that drug release could be controlled by the photothermal effect, which could be achieved at the tumor site (pH = 6.5) with laser irradiation on/off. Thus, this controlled release manner would significantly enhance biosafety *in vivo*.

To better investigate cellular uptake, CS NPs were labeled with rhodamine B-CS-RB NPs, which could be observed by confocal microscopy (Figure 3B, 5D and 5E). CAL 27 cells or HUVECs were cocultured with 5 µg/mL CS-RB NPs (concentration equal to sorafenib), which was observed for 24 hours. As shown in Figure 5D, the highest red signal in CAL 27 cells could be observed at 12 h, while a high signal could be observed at 6 h - 8 h, where there were no significant differences. Then, the uptake of CS-RB NPs in HUVECs was exhibited in a time-dependent manner at 6 h, which indicated that the maximum therapeutic effect would be achieved after at least 6 hours of incubation (Figure 5E).

Based on the uptake results, intracellular ROS generation was detected by using a DCF probe 8 h after incubation with 10 µg/mL CS NPs. Figure 5F and 5G show that with laser irradiation (808 nm, 0.5 W/cm²), an intense green signal was observed in CAL27 cells and HUVECs. In contrast, a weaker ROS signal was emitted without laser or CS NPs (Figure 5F and 5G). These results indicated that CS NPs were potent for PDT in OSCC.

Colocalization In Cells

To further study the location of CS-RB NPs, LysoTracker and MitoTracker probes were used to mark lysosomes and mitochondria, respectively. CAL 27 and HUVECs were incubated with CS-RB NPs for 8 h, after which they were observed by confocal microscopy. Additionally, colocalization was calculated via Pearson's coefficient. The results revealed that the red signal of CS - RB NPs was considerably merged

with MitoTracker with 91.8% and 81.2% Pearson's coefficient in CAL 27 and HUVECs, respectively (Figure 6C and 6D). In addition, the Pearson's coefficient values of CS-RB NPs located in lysosomes were 68.61% and 74.3% in CAL 27 and HUVECs, respectively, which were significantly lower than those in mitochondria (Figure 6A and 6B). The intracellular location of CS NPs would be beneficial for the effect of PDT. With laser-controlled drug delivery, CS NPs can achieve fairly precise local treatment, which contributes to maximizing the protection of normal tissue and limiting side effects.

In vitro Therapeutic effect

Given the detailed evaluation of the physicochemical properties and functions of CS NPs, studies at the cellular level were organized to investigate the therapeutic efficiency and biosafety. Medium supplemented with 5 µg/mL CS NPs was cocultured with CAL 27, HUVECs and human normal keratinocytes (HACAT) cells for 72 h to investigate whether CS NPs would affect the growth of the three types of cells. Cell viability of CAL 27 cells, HUVECs and HACAT cells treated with different concentrations of C NPs and CS NPs. C NPs alone had low cytotoxicity for all three types of cells, with a cell viability higher than 90%, even at a concentration of 60 µg/mL (Figure 7A). In contrast, with the assistance of 808 nm laser irradiation, the cytotoxicity obviously increased, leading to ~ 40% and more than 50% cell death at a 7.5 µg/mL concentration treated with C NPs and CS NPs, respectively. (Figure 7B: CAL 27 and HUVECs). Moreover, the cytotoxicity was much lower in HACAT cells, which was probably attributed to less targeting of sorafenib (Figure 7A and 7B in HACAT groups). This was a good way to protect healthy and normal tissues. Consequently, the combination of chemotherapy and PDT/PTT could significantly increase the killing effect and achieve satisfactory therapeutic outcomes.

It is widely recognized that sorafenib possesses dual antitumor activity, which can not only inhibit the proliferation of tumor cells directly by impeding the RAF/MER/ERK pathway but also interfere with angiogenesis by inhibiting the expression of vascular endothelial growth factor receptor (VEGFR). Therefore, to better reveal the process of vascular restriction, vessel tube formation and antiangiogenesis were conducted *in vitro*. As shown in Figure 7C, vessel tube formation was completed 6 h after induction, and 10 µg/mL sorafenib and CS NPs were added to the medium. During the following 6 h, vascular structures gradually decomposed, yet more vascular fractures could be witnessed in the CS NP group than in the sorafenib group. Additionally, with the combined use of laser, less than 10% vessel structure could be observed, indicating that the laser plus CS NPs played important roles in tumor vascular therapy.

Owing to the high proliferation of tumor cells, cell growth curves, colony formation assays and cell apoptosis assays were conducted to evaluate the effect of CS NPs. As shown in Figure 8A, compared with the control, CS NPs significantly reduced the growth of CAL 27 and HUVECs. However, the growth of HACAT cells was not affected by CS NPs compared with CAL 27 and HUVECs. Then, 2000 cells were seeded into wells to form cell colonies. However, after treatment with 10 µg/mL sorafenib or CS NPs for 72 h, cell proliferation decreased as the quantity of crystal violet spots decreased significantly, in which CS NPs displayed stronger cell restraints. As expected, with 3 laser irradiation cycles (0.3 W/cm², 10 min),

few CAL 27 or HUVECs survived and subsequently formed cell clones (Figure 8B). Moreover, an Annexin V – FITC/PI kit was applied to detect cell apoptosis with different treatments. As displayed in Figure 8C and 8D, sorafenib alone caused a small proportion of cell apoptosis, mainly at the early apoptotic stage, while in the CS NP groups, there was more apoptosis (>20% at the early stage and >12% at the late stage). With the combination of laser irradiation, chemotherapy, PDT and PTT were simultaneously achieved, which resulted in over 70% cell death. Consequently, these results indicated the effectiveness of the combination therapy strategy.

Nir-ii Fluorescence Of Cs Nps

To detect the depth penetration of NIR-II fluorescence, fresh chicken breast meat was used to simulate different thicknesses of physiological tissues (2 mm, 4 mm, 6 mm, 8 mm and 10 mm). The spatial resolution was evaluated through the full width at half-maximum (FWHM) of capillary tubes (0.5 mm). The gray values of all NIR-II images were normalized by ImageJ-win64. Then, gray value statics were fitted with the Gauss function to estimate FWHM via Origin software. As shown in Figure 9A, the fluorescence image of CS NPs could best simulate the shape and width of capillaries covered with 2 mm tissue (FWHM=0.75 mm, SBR=5.92). An increased FWHM could be observed along with an increased penetration depth, in which 8 mm was the ultimate thickness of penetration.

Furthermore, microvascular imaging was performed on the hindlimbs of nude mice *in vivo*. Before that, the Hemolysis assay was conducted. As shown in Figure S5, no hemolysis was observed. Therefore, CS NP solutions were regarded to be safe enough for nude mice. After injection of 10 µg/mL CS NP solution for 5 min, the blood vessels in the hindlimb could be observed, where the middle of the hindlimb blood vessel was picked to calculate the FWHM and SBR (Figure 9C and 9D). Based on the SBR and FWHM, 10-20 min was considered the best observation time window for hindlimb vessels after the injection, of which the SBR was 2.42, 2.56 and 3.34, respectively. In addition, the FWHMs of the hindlimb vessels at the same position were 0.60 mm (10 min), 0.64 mm (15 min) and 0.57 mm (20 min), illustrating the better bioimaging performance between 10-20 min.

According to the above results, tumor imaging *in vivo* was conducted to clarify the image rendering capability of CS NPs. Three positions were captured (Figure 9E). Then, long live NIR-II fluorescence was monitored for 12 h. Tumor imaging of CS NPs showed good pharmacokinetics and high quantum yield to afford high tumor accumulation and signals (Figure 9F). Compared with ICG-based NIR-I imaging, much more fluorescent interference in ICG-based imaging was observed, which made it difficult to distinguish the tumor count. In addition, the liver showed a very strong fluorescent signal, which further enhanced the difficulty of identifying the tumor site (Figure 9G). In this study, we observed the accumulation of CS NPs in the tumor site for 4 h. More interestingly, a signal of a small spot was observed for 12 h, which was along with the vessel co-option and close to the tumor (Figure 9F). The spot was subsequently confirmed to have micrometastasis by pathological examination (Figure 9H). These features were of great significance for clinical navigation during surgery. Surgeons could clearly evaluate the real boundary of

tumors in real time during surgery. More importantly, they could also discover micrometastasis as early as possible by image-guided tracking of vessel co-option. In particular, the connection (the vessel co-option) appeared to be a tunnel between the adjacent normal tissues and tumors.

Antitumor therapy in vivo

Based on the satisfactory outcome at the cellular level, *in vivo* experiments were then performed to further investigate the therapeutic outcome of CS NPs. In this experiment, CAL 27 tumor-bearing mice were randomly divided into 5 groups receiving different treatments (Control, Sorafenib, CS NPs, C NPs + Laser and CS NPs + Laser). In addition, vessel co-options were tracked by NPs and included in therapies. During the 25-day therapy process, tumor-bearing BALB/c mice in all groups, except the sorafenib group, manifested an increase in body weight, demonstrating the good biosafety of NPs (Figure 10A). Compared with the control group, limited suppression of tumor growth was observed in the sorafenib group, which could be ascribed to the limited drug accumulation inside the tumor and the clearance effect. However, mice treated with CS NPs exhibited a remarkable tumor inhibition effect, especially mice treated with CS NPs + 808 nm laser irradiation. This result revealed that CS NP-mediated vascular blockade combined with PDT and PTT could acquire an ideal therapeutic effect (Figure 10B). Furthermore, the survival of mice in all groups was analyzed. As shown in Figure 10C, free sorafenib did not show a benefit in survival compared with the control, implying the high biological toxicity or low therapeutic effect of sorafenib. However, loading NPs, CS NPs and CS NPs + laser treatment significantly prolonged survival, which was consistent with the results at the cellular level. Subsequently, intact tumor and main organ specimens were obtained. The H&E results of tumors are shown in Figure 10E, which shows poorly differentiated squamous cell carcinoma.

To further evaluate the therapeutic efficacy, the expression levels of CD31, VEGFR and TUNEL in the tumor site were calculated. As displayed in Figure 11A, different degrees of vascular fractures appeared alongside the vessel co-options, which were located at the frontier of the OSCC tumor. The frequency of vascular fractures (white arrowhead) in the sorafenib groups was obviously lower than that in the CS NPs and CS NPs + laser groups. The CS NPs + laser group showed the most elevated frequency of vascular fractures, suggesting that the combination of NPs and laser holds high proficiency in the vessel blockade effect (Figure 11A). In addition, CS NPs + laser treatment exhibited the best antitumor outcome regarding cell apoptosis induction and remarkable VEGFR inhibition (Figure 11B and 11C). All these data indicated the outstanding performance of the combination of vessel blockade with PDT and PTT in OSCC tumor therapy.

The main organs of mice were obtained and stained with H&E. As shown in Figure S6, damage to the livers in the sorafenib group could be witnessed. Red arrows indicate vacular degeneration and necrosis of hepatocytes. Moreover, foam-like structures could also be observed in liver specimens. In contrast, no abnormalities or toxic representations were observed in the other groups, indicating the excellent biosafety of NPs.

Biocompatibility and blood pharmacokinetics evaluation in vivo

Owing to the side effects of sorafenib, the biocompatibility of CS NPs was explored. First, a blood pharmacokinetics experiment was conducted. Six rats (~ 130 g) were randomly divided into two groups. Rats received gavage administration of sorafenib or CS NP solution at a concentration of 2.5 mg/kg. Before that, the standard curve of sorafenib solution was evaluated by UFLC-QTRAP-MS/MS (Figure 12A). Then, blood samples from rat veins at different time points were examined. The results in Figure 13B and 13C indicated that the blood sorafenib concentration in the CS NP groups was significantly higher than that in the free sorafenib group. After 2 h of gavage, the accumulation amount of CS NPs reached a maximum in the blood. The half-life time of CS NPs ($t_{1/2} = 10.65\text{h}$) was more than 10-fold that of free sorafenib ($t_{1/2} = 0.83\text{h}$). Low blood concentration and low half-life time would explain the low therapeutic effect of free sorafenib in clinical practice. Based on the half-life time, rats received 5 gavage administrations.

As shown in Figure 12D to 12I, the function of the liver and kidney indicated that gavage administration of C NPs and CS NPs did not cause any obvious disturbance to the hematological indexes, further supporting the good biocompatibility of NPs. However, sorafenib caused significant damage to liver function, which was in accordance with the previous H&E result in Figure S6. To further investigate the biocompatibility of these drugs, acute toxicity tests and dermal sensitization responses were added to KM mice and Sprague–Dawley rats (SD rats), respectively. As shown in Figure S8, sorafenib resulted in cardiotoxicity, decreased skin thickness and skin structural disorder, while no significant damage was seen in the CS NP or C NP groups. Therefore, nanotechnology was an effective and uncomplicated method to reduce the toxicity of sorafenib.

Antitumor Immune Effects Induced By Combination Therapy

Given that CS NP-mediated vascular therapy and PDT/PTT could almost eliminate the tumors and inhibit metastasis, a dual-tumor model (primary and metastatic tumors) in C3H mice was established to further study the antitumor effect. The experimental procedure is shown in Figure 13A. After 5 primary therapies, a metastasis model was established in the opposite site. As expected, the growth speed of metastatic tumors was different in various groups. Notably, sorafenib alone and NPs not only suppressed the primary tumor but also inhibited the metastatic tumor (Figure 13B and 13C). In addition, CS NPs + laser yielded the highest inhibitory effect on metastatic tumors. All data suggested that vascular blockade, PDT and PTT have the potential to stimulate antitumor metastasis immune effects to eliminate distant metastasis. Therefore, effector T cells of antitumor immunity were assessed. CD8⁺ T cells, which can directly attack tumor cells, and CD4⁺ T cells also play a crucial role in antitumor immunity. The populations of CD3⁺ and CD4⁺ T cells and CD3⁺ and CD8⁺ T cells in the blood were detected by flow

cytometry. As shown in Figure 13D, all treatments induced a systemic immune response to cytotoxic T cells (CD8⁺) and helper T cells (CD4⁺). Furthermore, it was found that with the delivery of NPs, the antitumor immune effect increased significantly. In particular, in the CS NPs + laser group, CS NPs played an important role in triggering systematic immunity for cancer therapy. In the mouse model, combination therapy of blood blockade and PTT and PDT effectively increased the ratio of antitumor/protumor immune cells and significantly inhibited metastatic tumor growth, and the efficacy of combination therapy was superior to that of monotherapy.

Conclusion

In this work, we successfully developed novel NIR-II therapeutic agent CS NPs for chemotherapy/PDT/PTT combination treatment, which achieved a win–win outcome by tracing and blocking tumor-associated vessels for oral squamous cell carcinoma. The A-D-A structure of COi8DFIC contributed to strong luminescence, with the PLQY value of C NPs achieving 1.19%. In addition, the obtained CS NPs possessed a high penetration depth of 6 mm and a high SBR of 4.3, which was beneficial for microvessel imaging. *In vivo* optical imaging results revealed that CS NPs could track vessel co-option and accurately locate tumor micrometastasis in a timely manner, which was significant for clinical navigation. By tracking and blocking vessel co-option, CS NPs + laser exhibited effective tumor suppression with outstanding biosafety and organ protection. Our results demonstrated that the combination therapy not only pruned blood vessels, which are essential to cancer growth and metastasis but also stimulated the tumor immune response. Therefore, CS NPs would bring promise for clinical translation, and the proposed strategy may provide a win–win strategy for OSCC treatment.

Declarations

Competing interests

There is no competing interests in the research.

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Availability of data and material

The processed data required to reproduce these findings are available to download from the official website of the published article.

Consent for publication

The authors agreed to publication in the journal.

And authors confirmed:

That the work described had not been published ever;

That its publication had been approved by the responsible authorities at the institution where the work was carried out.

Ethics approval and consent to participate

Patient samples and relative statistics were all approved by the patients and the Research Ethics Committee of Nanjing Stomatological Hospital (NJSH-2021NL-019). All animal experiments were approved by the Animal Ethical and Welfare Committee of Nanjing University (IACUC—D2102071).

Authors' contributions

Zheng Wei: Conceptualization, Investigation, Data curation, Writing- Original draft preparation, Funding acquisition. Hongbo Zhang and Huihui Zou: Validation, Visualization, Writing - Original Draft. Chuanhui Song: Methodology, Investigation, Data Curation. Sufeng Zhao: Visualization, Methodology, Formal analysis. Guorong Zhang: Software. Zichen Cao: Visualization. Xinyu Zhang: Investigation. Yu Cai: Supervision, Writing - Review & Editing, Funding acquisition. Wei Han: Supervision, Conceptualization, Supervision, Project administration, Funding acquisition.

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Figures

Figure 1

A. Blood vessels in the healthy tissue. B. Vessel co-options exist between tumor and nonmalignant tissues. C. Angiogenesis inside the tumor. D. Normal oral mucosa epithelium cells and blood vessels. E. Vessel co-options in the OSCC tumor frontier adjoining the nonmalignant tissue (indicated by the red arrow). F. Angiogenesis in the center of OSCC.

Figure 2

A. The H&E staining of one typical case of OSCC. B. The CD31 staining of OSCC vascular vessels. The circle marked zone was the area of interest. B (a-e) showed all 5 types of WPOI in OSCC. C. The relationship between vessel co-option MVD and WPOI. They had a significant correlation based on the Pearson correlation test ($p < 0.001$). D. The relationship between vessel co-option MVD and T stage ($p < 0.05$).

Figure 3

A. Absorption spectra of COi8DFIC (black line) and C NPs (red line). B. Absorption spectra of Sorafenib, C NPs, CS NPs and CS NPs-Rhodamine B. C. The photo stability of ICG, COi8DFIC and CS NPs with 808 nm laser irradiation. D and E. The spherical morphology of C NPs and CS NPs characterized by TEM. In addition, the nano size calculated by DLS. F. The long-term stability in simulated physiological environments (PBS, DMEM and FBS, respectively). G. The fluorescence spectra of COi8DFIC, C NPs and CS NPs. H. The PLQY of NPs was calculated. IR – 26 was as the reference (0.05%). I. NIR-II fluorescence images of CS NPs stored for 7 days in RT.

Figure 4

A (a-c). The photothermal effect of CS NPs. (a) Photothermal effect of CS NPs at various concentrations (1 W/cm^2). The concentration of CS NPs was equivalent to that of COi8DFIC. (b) Photothermal performance under different power of 808 nm irradiation (10 $\mu\text{g/mL}$). (c) Photothermal stability of CS NPs during heating-cooling cycle (3 cycles performed). B (a-c). The total ROS generation of COi8DFIC irradiated at 660 nm. (a) The total ROS produced by MB with 8 seconds 660 nm at the power of 0.3 W/cm^2 . DPBF probe was utilized as which could be decomposed when ROS materials existed. (b) The total ROS generated by COi8DFIC with same circumstance as MB. (c) The linear fitting of the DPBF absorption and irradiation time. The ROS quantum yield of Coi8DFIC was 18.85 (MB was 0.57). C (a-c) The SOS production of ICG and CS NPs irradiated at 808 nm (0.5 W/cm^2). SOSG probe was used to capture SOS. (a, b) the fluorescence intensity profiles of ICG and CS NPs irradiated with 808 nm laser,

respectively. (c) The linear fitting of SOSG fluorescence intensity at 525 nm and irradiation time. The slope of these lines was adopted to calculated the quantum yield.

Figure 5

A. Size of CS NPs (20 µg/mL) decreased after 808 nm laser (0.3 W/cm^2) irradiation. Since embedding rates of COi8DFIC and Sorafenib were similar, the concentration of sorafenib was used to represent the concentration of CS NPs in the subsequent experiments. B and C. showed the Sorafenib release in different pH (7.4, 6.5, 5.3) and temperature environment (37 °C, 45 °C and 50 °C), respectively. 20 µg/mL CS NPs was utilized to conduct the dialysis experiment. 1000D of molecular weight cutoff and 45 mm of flatten width was applied in dialysis bag. The study was observed for 20 min to stimulate the PDT and PTT *in vivo* (*: $p < 0.05$, ***: $p < 0.0001$). D and E showed the cellular uptake of CS - RB NPs in CAL 27 and

HUVECs. The fluorescence signal of CS - RB NPs was captured by confocal microscope (Scale bar = 20 μm). F. CLSM analysis of ROS generated by CAL 27 and HUVECs under in conditions (Scale bar = 50 μm , laser: 808 nm, 0.3 W/cm²). DCFH-DA was used as the detection probe.

Figure 6

A and B. CAL 27 and HUVECs were marked by LysoTracker with green fluorescence. C and D showed the colocalization of CS – RB NPs (Red) and MitoTracker (Blue) (Scale bar = 20 μm). All (b) figures were the Pearson's coefficient calculated by ImageJ.

Figure 7

A and B. Cell viabilities of CAL 27, HUVEC and HACAT cells in different conditions with varied C NPs and CS NPs concentration (laser: 808 nm, 0.3 W/cm^2 , 10 min) (mean $\pm$ SD, $n = 6$). C. Vessel formation and anti-angiogenesis *in vitro*. Scale bar = 500 μm .

Figure 8

A. Cell proliferation of CAL 27, HUVEC and HACAT treated with control or CS NPs. B. Cell colon formation and inhibition of CAL 27 and HUVECs. Live cell colons could be stained by hexamethyl pararosaniline in blue. C. Apoptosis detection of CAL 27 cells with various treatment. D. Apoptosis detection of HUVECs with various treatment. Q1 (Annexin V⁻/PI⁻): live cells, Q2 (Annexin V⁺/PI⁺): late-stage apoptotic cells, Q3 (Annexin V⁺/PI⁻): early-stage apoptotic cells.

Figure 9

A. Penetration depth of CS NPs in the simulated physiological tissue model. B. The fluorescence intensity calculated with grave value of the images. C. The micro vascular imaging *in vivo* at different time after injection. D. The middle of hindlimb blood vessel was picked to calculate the FWHM and SBR. The FWHM and SBR were marked around the fluorescence profiles. E. OSCC tumor imaging at right posterior dorsal with three positions. Red arrows indicated the vessel co-option, which appeared to be a tunnel between

tumor and nonmalignant tissue. F. Long live tumor imaging of CS NPs. Green arrows marked the micrometastasis and red arrows prompted the vessel co-option. G. The comparison of tumor imaging between ICG and CS NPs. H. The pathological result of the micrometastasis.

Figure 10

A. Body weight of mice in various groups; B. Tumor volume. C. Survival analysis of various groups during 25-day therapy. (control: blue; Sorafenib: red; CS NPs: green; C NPs + Laser: purple: CS NPs + Laser: orange). D. OSCC tumors in all groups. ●: The tumor disappeared. ⊗: Death of nude mice. E. Tumor H&E results of all groups. One nude mouse in C NPs group had tumor metastasis. Scale bar = 100μm. F. Mice photographs after 25-day treatment.

Figure 11

A. The CD 31 immunohistochemical staining in the vessel co-option areas in the frontier of tumor. White arrows noted the vascular fractures. B. VEGFR expression in tumors. The brown staining was positive expression of VEGFR. C. The TUNEL in tumors. Green spots were death cells. Scale bar = 100 μ m.

Figure 12

A. The standard curve of the sorafenib concentration. B and C. Sorafenib concentrations of blood in rats received gavage administration with free sorafenib and CS NPs. D-F. The liver function of rats in various groups. G-I. The kidney function of rats in various groups. The concentration equaled to the Sorafenib at 2.5 mg/kg.

Figure 13

CS NPs induced phototherapy enhanced systemic antitumor immunity effect against metastasis tumors. A (a) and (b) showed the schematic instruction of treatment. B. Metastasis tumors of all treatment groups. "X" represented the death of the mouse. C. Volume of metastasis tumor after different treatment. (n = 4, mean ± SD). D. The CD 8 positive (CD 8⁺) and CD4 positive (CD 4⁺) cell populations in the blood of SCC7 bearing tumor mice induced by different treatments. The marker of CD 3 was employed to select T

cells at first. E. Quantitative analysis of T cell populations in each treatment group. (n = 4, mean $\pm$ SD). *p < 0.05; **p < 0.01; ***p < 0.001.

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