Biomimetic Manganese-Eumelanin Nanocomposites for Combined Hyperthermia-Immunotherapy Against Prostate Cancer

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Abstract

Pro-tumoral and immunosuppressive M2-like tumor-associated macrophages (TAMs) contribute to tumor progression and distal metastasis. However, current TAMs-modulating therapeutic strategies often encounter challenges including insufficient immune activation, weak antigen presentation ability and unsatisfactory antitumor immune performance. Herein, cyclic RGD peptide functionalized and manganese doped eumelanin-like nanocomposites (RMnMels) were designed for combined hyperthermia-immunotherapy against PC3 prostate cancer. The RMnMels could promote M2-to-M1 macrophage repolarization *via* scavenging reactive oxygen species and remodel the tumor immunosuppressive microenvironment (TIME). Following near-infrared light irradiation, RMnMels-mediated local hyperthermia not only could destroy tumor cells directly through thermal ablation, but also elicit the release of damage associated molecular patterns and tumor-associated antigens, which could act as effective immunological stimulators to provoke robust tumor immunogenicity and strong antitumor immune responses. The results showed that RMnMels could effectively scavenge reactive oxygen species and promote M2-to-M1 macrophage repolarization both *in vitro* and *in vivo*. Synergistically enhanced anti-tumor therapeutic efficacy was achieved following single administration of RMnMels plus single round of laser irradiation, evidenced by decreased primary tumor sizes and decreased number of distant liver metastatic nodules. The as-developed RMnMels may represent a simple and high-performance therapeutic nanoplatform for immunomodulation and enhanced antitumor immune responses.

1. Introduction

Tumor immunosuppressive microenvironment (TIME) is one crucial driver to promote tumor genesis, progression, metastasis, immune evasion, and therapeutic resistance.[1, 2] As one of the most abundant components of immune cells infiltrating the tumor microenvironment, tumor-associated macrophages (TAMs) can be actively recruited to tumor sites, and secrete various cytokines and chemokines which contribute to tumour immune evasion, resulting in immunotherapy failure.[3-5] TAMs are typically differentiated into anti-tumoral M1-like and pro-tumoral M2-like phenotype according to their polarization states.[6, 7] Usually, TAMs predominately present as M2-like phenotype, and the M2-dominated TIME is responsible for a variety of tumor-promoting activities at both primary and metastatic tumor sites.[8-11] Up to now, several TAMs-modulating therapeutic strategies have been explored as emerging paradigms.[1, 2, 12] However, one main drawback of depleting TAMs is that macrophages may lose their potential immunostimulatory effects as phagocytes and professional antigen-presenting cells.[13] It is of great interest to develop novel approaches for promoting the anti-tumor functional states of TAMs and enhancing the antitumor therapeutic efficacy.

Interestingly, TAMs are characterized by high plasticity, allowing for significantly different functions following interaction with microenvironmental cues under specific conditions.[4, 14, 15] As is well-known, tumor microenvironment is highly oxidative and characterized by overburdened reactive oxygen species (ROS). Previous studies revealed that ROS were crucial signaling molecules in M2 macrophage

polarization and function.[16] M2 macrophages are more susceptible to ROS variation compared with M1 macrophages.[17] The M1 macrophages are effective effector cells for direct tumor cell killing through extensive release of various cytokines and local recruitment of pro-immunostimulating macrophages.[13] In this regard, selective M2-to-M1 macrophage repolarization has drawn significant attention for alleviating immunosuppression and improving antitumor responses.[18, 19] Recently, a variety of therapeutic agents (*e.g.*, monoclonal antibodies, small molecular inhibitors, and complicated nanoparticles) have been extensively explored for M2-to-M1 macrophage repolarization.[2, 16, 18-21] Despite promising, these approaches encounter problems such as insufficient immune activation, weak antigen presentation ability, and unsatisfactory antitumor immune response.[22, 23] It is highly reasonable to integrate other appropriate combination strategies for improved therapeutic efficacy.

As an emerging therapeutic modality, photothermal monotherapy seems to be insufficient in tumor eradication attributed to low tissue penetration and the presence of TIME. Recently, combined hyperthermia-immunotherapy has received explosive attention as a novel synergistic antitumor regimen for expanding the attack area that are unaccessible to the triggering light, including residual tumor deep regions, disseminated metastasis and recurrent tumors.[24] In this scenario, photothermal conversion agents mediated local temperature increase at tumor sites could induce the release of multiple tumor-associated antigens and endogenous danger signals *in situ*, which could elicit the activation of dendritic cells and tumor antigen presentation to T cells, resulting in improved tumor immunogenicity and amplified systemic antitumor immune responses.[25-27] Nowadays, many nano-agents with excellent photothermal conversion efficiency and satisfactory photothermal stability have been reported.[28] However, the majority of them are unable to be implemented in clinic attributed to the complicated and time-consuming fabrication procedures and possible long-term safety concerns.[29] Developing biomimetic nanoplatfroms derived from naturally components *via* convenient procedures is highly beneficial, because they can be easily and gradually decomposed into nontoxic small molecular metabolic components.[30] Melanins are natural occurring biopolymers distributed in various parts of living organisms, possessing excellent biocompatibility and biodegradability.[31] Notably, owing to the abundance in reductive functional groups, melanins exhibit broad-spectrum radical scavenging capacities,[31] which render them promising as novel potent antioxidative nanoplatfroms. Here, we report cyclic RGD peptide functionalized and manganese doped eumelanin nanocomposites (RMnMels) for high-performance and combined hyperthermia-immunotherapy against PC3 prostate cancer (**Scheme 1**). After intravenous injection, the targeting peptide could facilitate preferential intratumoral accumulation of RMnMels *via* specific ligand-receptor interaction. Then, RMnMels could promote M2-to-M1 macrophage repolarization *via* scavenging ROS *in situ*. Following near-infrared light irradiation, RMnMels-mediated local hyperthermia could destroy tumor cells directly, and the released damage associated molecular patterns and tumor-associated antigens could act as effective immunological stimulators to provoke robust tumor immunogenicity and strong antitumor immune responses. These together could synergistically reverse the TIME and realize improved therapeutic efficacy. As expected, RMnMels showed satisfactory T_1 -weighted magnetic resonance/photoacoustic imaging performance for visualization of nanoparticles *in vivo*. Following single administration of RMnMels plus single round of laser irradiation,

remarkable anti-tumor efficacy was achieved, evidenced by decreased primary tumor sizes and decreased number of distant metastatic nodules in PC3 prostate cancer bearing mice. Taken together, the as-developed RMnMels may represent a simple and high-performance therapeutic nanoplatform for immunomodulation and enhanced antitumor immune responses.

2. Results And Discussion

2.1. Preparation and characterization of RMnMels

Mn(II) ions doped eumelanin-like nanoparticles (MnMels) were first prepared through a simple intra-polymerization doping strategy as we previously described with minor modifications.[32] Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) showed that RMnMels were uniform and monodispersed spherical in morphology, with a mean diameter of 80-100 nm (Figure 1a, 1b). Energy dispersive X-ray (EDX) element mapping analysis showed the presence and homogeneous distribution of Mn element, confirming the successful doping of Mn element inside the entire nanostructures (Figure 1c, 1d). The Mn loading efficiency was quantified as about 9.3% weight, determined by inductively coupled plasma mass spectrometry (ICP-MS). X-ray photoelectron spectroscopy (XPS) survey spectra showed that high-resolution Mn curve exhibited two binding energy peaks centered at 641.8 and 653.6 eV, which were assigned to Mn 2p_{3/2} and Mn 2p_{1/2} states, respectively. The peak centered at 646.0 eV was assigned to Mn 2p_{3/2} satellite peak (Figure 1e). It suggested the presence of a large proportion of Mn (II) species (Figure 1e, S3). To improve the circulation stability and tumor targeting capacity, PEGylated MnMels (PMnMels) and cRGD peptide modified MnMels (RMnMels) were synthesized. Fourier transform infrared spectroscopy (FTIR) results showed characteristic band signals for amide bond formation located at around 1650 cm⁻¹ and 1535 cm⁻¹, arising from the C=O and C-N stretching vibration (Figure 1f). Furthermore, RMnMels showed a peak at 1357 cm⁻¹ attributed to the sp²-bonded carbon atoms, almost the same as natural eumelanin. These results indicated successful synthesis of RMnMels and PMnMels. Given the paramagnetic property of Mn(II) ions, the feasibility of RMnMels as magnetic resonance (MR) contrast agents were evaluated. *T*₁-weighted images (*T*₁WI) demonstrated a positive contrast enhancement in a concentration-dependent manner and the longitudinal (*r*₁) relaxivity was estimated to be 2.48 mM⁻¹s⁻¹ (Figure 1h). After injection into PC3 prostate cancer bearing mice, both PMnMels and RMnMels provided positive contrast enhancement on *T*₁WI (Figure 1g). At 6 h post-injection, PMnMels and RMnMels were prominently accumulated in tumors, and showed maximal signal intensities. Benefitted from peptide mediated active targeting, RMnMels accumulated at the tumor site more efficiently, which was about 10 times higher than PMnMels (Figure 1i). The concentration-dependent optical absorption of RMnMels indicated their potential in photoacoustic imaging applications (Figure S6). As expected, RMnMels generated bright photoacoustic signals, and the intensities were in linearity with nanoparticle concentrations (Figure 1k). The photoacoustic signal intensity at tumor sites reached maximum at 6 h post-injection (Figure 1j), in accordance with MRI results. At 6 h post-injection, RMnMels showed more efficient intratumoral accumulation and produced stronger signal intensity, which was about 2.4 times higher than that of

PMnMels (Figure 1l). The results indicated great potential of RMnMels for T_1 -weighted MR/photoacoustic dual-modal imaging *in vivo*.

Nanoparticles showed negligible cytotoxicity on PC3 and RAW 264.7 cells in the range of testing concentrations (Figure S7), indicating their biosafety and potential for *in vivo* applications. Cellular TEM images showed that RMnMels were located in endosome-like structures (Figure S9a), indicating efficient cellular internalization. There was no obvious absorption variation in the near infrared (NIR) region after modification (Figure 2a). There was a good linear correlation between the absorbance at 690 nm and mass concentrations (Figure 2b), indicating their potential in hyperthermia applications. Attributed to the strong NIR absorption, the temperature of RMnMels solution increased in laser power density/concentration dependent manners (Figure 2c, 2d, S11). The hyperthermia-mediated cell killing effect of RMnMels was verified by Calcein-AM/PI co-staining (Figure 2j). Following RMnMels plus laser irradiation, remarkably decreased PC3 cell viability with increased incubation concentrations was determined. The quantitative results demonstrated that the therapeutic efficacy was associated with nanoparticle concentrations (Figure 2k). Then, the antioxidative activity of RMnMels toward multiple free radicals was investigated.

As shown in Figure 2e, RMnMels exhibited concentration-dependent scavenging capacity against 2,2-di-(4-tert-octylphenyl)-1-picrylhydrazyl radical (DPPH). The scavenging activity of RMnMels against superoxide anion ($O_2^{\cdot-}$) was confirmed by electron spin resonance (ESR) spectroscopy (Figure 2f). The reduced signal of paramagnetic adduct DMPO-OH was resulted from the reaction between DMPO and hydroxyl radical ($\cdot OH$), indicating that RMnMels could scavenge $\cdot OH$ effectively (Figure 2g). The intracellular ROS levels in RAW 264.7 cells following different treatments were detected. RMnMels treatment resulted in the most significantly decreased intracellular fluorescence intensity (Figure 2m, 2l), indicating their effective ROS scavenging. The ROS levels in resected tumor tissues were verified by immunofluorescence (Figure 2n). Following RMnMels plus laser treatment, the most remarkably decreased ROS levels were observed within the tumor tissues. Given the strong ROS scavenging capacity of RMnMels, their effect on mitochondrial function of PC3 cells was measured by a cellular energy metabolism analyzer. The results showed that RMnMels had a negative effect on oxygen consumption rate (OCR) (Figure 2h). PC3 cells incubated with RMnMels showed a decreased spare respiration by $12.53 \pm 3.65\%$ ($P < 0.05$, Figure 2i). The results suggested that RMnMels could significantly inhibit mitochondrial function and alleviate the oxidative stress in PC3 cells.

2.2. RMnMels promote M2-to-M1 macrophage repolarization

Inhibiting M2 macrophages is considered as a promising cancer therapeutic approach[19]. The direct repolarization efficacy of RMnMels on M2 macrophages was analyzed *in vitro*. RAW264.7 cells were first pre-stimulated with IL-4 to generate M2-like phenotype, and then co-incubated with RMnMels for another 24 h. Real-time polymerase chain reaction (RT-PCR) results revealed that RMnMels treatment up-regulated M1-related markers and down-regulated M2-associated markers (Figure 3a). Compared with PBS control,

RMnMels treatment significantly up-regulated the mRNA levels of iNOS, TNF- α , and CD86 (M1-related markers), in which the iNOS increased as much as 10 times. Western blot assay showed that RMnMels treatment induced significant up-regulation of TNF- α , IL-1 β and iNOS protein expression levels in M2 macrophages (Figure 3b). Flow cytometric analysis showed that RMnMels treatment induced remarkably increased proportions of CD86^{high} M1 macrophages (Figure 3c).

Notably, RMnMels treatment induced M2 macrophages repolarization was similar to that of 10 ng/mL lipopolysaccharide (LPS) stimulation, which could skew M2 macrophages to M1 phenotype after more than 24 h incubation. These phenomenon were further confirmed by cell immunofluorescence assay. M1 marker iNOS expression increased and M2 marker CD163 expression decreased following RMnMels treatment (Figure 3f). Moreover, the levels of inflammatory cytokines following different treatments in RAW 264.7 cells were further verified by ELISA. The results showed that RMnMels treatment induced the upregulation of several M1 markers including IL-6, TNF- α , and IL-1 β , and inhibited M2 markers IL-10 production (Figure 3d). These results provided evidence that RMnMels were capable of driving the polarization of TAMs from M2-like toward M1-like phenotype.

2.3. RMnMels significantly inhibit tumor growth and alleviate the TIME

Considering the potential of hyperthermia for improving tumor immunogenicity, whether RMnMels-mediated hyperthermia could trigger strong immune responses to recognize tumor-specific antigens was evaluated. The time schedule for treatment is shown in Figure 4a. The immunological effects of thermal ablation was first tested in PC3 tumor bearing mice. The temperature at the tumor sites increased to about 45.0°C following RMnMels plus laser treatment. However, for mice receiving laser only, the temperature maintained at about 36.4°C, which was insufficient to elicit any tissue damage (Figure 4b, 4c). Besides directly destroying tumor cells, RMnMels-mediated hyperthermia could serve as an efficient immune stimulator to reverse the TIME and promote systemic antitumor immunological effects. Tissue immunofluorescence assay showed remarkably decreased M2 macrophages at the tumor sites following RMnMels and RMnMels plus laser treatment (Figure 3g). Next, the serum levels of typical inflammatory cytokines were detected, given their roles as indicators of immunological responses. Consistent with cellular ELISA results, a similar shift in production of inflammatory cytokines *in vivo* was detected (Figure 3e), which could contribute to enhanced immune responses.[33] In the RMnMels only group, local hyperthermia effect was excluded due to the absence of laser irradiation, and the moderate changes of cytokine levels was attributed to RMnMels. Under the synergism of RMnMels and NIR irradiation, the IL-6, TNF- α and IL-1 β levels further increased and remained within the normal ranges, indicating safe and efficient immunological responses. Collectively, these results revealed that RMnMels could significantly alleviate the TIME by inhibiting M2 macrophage polarization *in vivo*. The antitumor effect was moderately ameliorated following RMnMels only treatment, benefitting from their capacity in immunostimulation. PMnMels plus laser treatment had a similar antitumor effect because of inadequate intratumoral accumulation and subsequent restrained photothermal efficacy.

Benefitted from specific tumor accumulation *via* peptide mediated active-targeting, RMnMels plus laser treatment showed the strongest photothermal effect and immunogenicity, and thus maximized therapeutic efficacy. The synergism of immunostimulation and hyperthermia was evidenced by decreased tumor proliferation, resulting in almost complete inhibition of tumor growth. RMnMels only, PMnMels plus laser and RMnMels plus laser treatment led to 38.6%, 85.0% and 96.6% reduction in tumor volume, respectively (Figure 4d). To further elucidate the antitumor mechanism of the prominent therapeutic performance, the profile of tumor-infiltrating immune cells was evaluated using flow cytometry (Figure S13). Compared with PBS only and PBS plus laser treatment, PMnMels plus laser treatment and RMnMels plus laser treatment significantly induced TAMs infiltration into tumor tissues (Figure 4e, 4g). The combination with TAM repolarization significantly decreased the M2-like TAMs and increased the M1-like TAMs in the RMnMels plus laser group (Figure 4f, 4h, 4i), resulting in an elevated M1/M2 ratio (Figure 4j). Correspondingly, the TIME was remodeled in the RMnMels plus laser group. The immunofluorescence and TUNEL staining also showed maximum M1-like TAMs and tumor cell apoptosis in the RMnMels plus laser group (Figure 3g, 5e).

2.4. RMnMels significantly inhibit tumor metastatic and Safety evaluation

In addition to significantly inhibited primary tumors, the minimum metastatic lesions in the liver and lymph nodules were observed in the RMnMels plus laser group (Figure 5c-d). The RMnMels plus laser treatment exhibited the most remarkable capability to inhibit tumor metastatic compared with other groups. Notably, H&E stained images of major organs in PC3 tumor-bearing mice (Figure S14) and serum biochemical both in tumor-bearing mice and healthy mice (Figure. 5f, S15) showed scarcely any abnormality. The results indicated satisfactory safety of RMnMels, which was suitable for *in vivo* bio-applications.

3. Conclusion

In summary, an immunomodulation-enhanced nanoplatform is constructed to realize synergistic hyperthermia-immunotherapy. Unlike conventional TAMs-modulating therapeutic strategies, the intrinsic nature of melanin endows the as-developed RMnMels with satisfactory biocompatibility and multifunctionality, and they could scavenge excess ROS and induce M2-to-M1 macrophage repolarization at tumor sites *in situ*, remodeling the TIME effectively. Following thermal ablation induced by local light, RMnMels could serve as immunological stimulators to improve the immunostimulatory efficacy and strengthen the antigen presentation ability. Our results demonstrated that high-performance hyperthermia-immunotherapy synergism was achieved following single administration of RMnMels plus single round of laser irradiation. This work will open up new insights for enhanced antitumor immune responses and improved tumor therapeutic efficacy.

4. Experimental Section

Materials

Potassium permanganate (KMnO_4), 3,4-Dihydroxy-DL-phenylalanine (DL-DOPA), propidium iodide (PI), and calcein acetoxymethyl ester (Calcein AM) were purchased from Aladdin Reagent (Shanghai, China). Thiol-terminated methoxy-poly (ethylene glycol) (mPEG-SH, molecular weight=5000 Da) was purchased from Seebio (Shanghai, China). Minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), and Fetal bovine serum (FBS) were purchased from Gibco (New York, USA). 2,2-di-(4-tert-octylphenyl)-1-picrylhydrazyl radical (DPPH) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cyclic RGD peptide was purchased from ChinaPeptides Co., Ltd. (Jiangsu, China). IL-4 were purchased from BioLegend (San Diego, USA). Lipopolysaccharide (LPS), ammonium hydroxide (30%), β -actin, and HRP-conjugated mouse monoclonal antibody were purchased from Sigma-Aldrich (St. Louis, USA). ELISA assay kits (TNF- α , IL-6, IL-10, IL-1 β) were purchased from MeiBiao Biological Technology (Jiangsu, China). CCK-8 assay kit was purchased from Beijing Solarbio Science & Technology (Beijing, China). ROS assay kit was purchased from Beyotime (Shanghai, China). Anti-CD163 and anti-iNOS were purchased from Abcam (Massachusetts, USA), CD86-PE and CD206-APC monoclonal antibodies were purchased from eBiosciences (San Diego, USA). COX-2 murine polyclonal antibody and Arginase-1 (Arg-1) murine monoclonal antibody was purchased from Proteintech (Chicago, USA).

Synthesis of MnMels, PMnMels and RMnMels

Mn (II) ions doped eumelanin-like nanoparticles (MnMels) were prepared through a facile and simple intra-polymerization doping strategy as we previously described.[32] For preparing PEGylated MnMels (PMnMels), MnMels and mPEG-SH (feeding mass ratio = 1:5) were vigorously stirred in pH=10 buffer solution for 12 hours at room temperature. The as-obtained PMnMels were purified by several times of centrifugation-redispersion processes (17500 rpm, 15 min) in deionized water to remove residual mPEG-SH. The cyclic-Arg-Gly-Asp (cRGD) peptide modified MnMels (RMnMels) were prepared *via* a Michael addition approach. Briefly, 2 mg/mL MnMels was added in 950 μL $\text{NH}_3\cdot\text{H}_2\text{O}$ (30%) solution (pH = 10). Then, 500 nmol cRGD in 50 μL water was added and reacted at 4°C for 12 h. The products were retrieved by four times of sequential centrifugation-redispersion processes (17500 rpm, 15 min) in deionized water to remove excess peptide. Finally, the as-obtained RMnMels were redispersed in deionized water for subsequent experiments.

Physicochemical characterization

The shape and morphology of nanoparticles were acquired by transmission electron microscopy (TEM, Tecnai G2 F30 S-TWIN, USA) and scanning electron microscope (SEM, Hitachi SU-70, Tokyo, Japan). Elemental mapping scanning images was acquired using energy-dispersive X-ray (EDX) spectroscopy equipped on TEM. The zeta potential and hydrodynamic sizes of nanoparticles were determined by dynamic light scatterer (DLS, Zetasizer Nano ZS90, Malvern Instruments, UK). The chemical functional groups were measured by fourier transform infrared spectroscopy (FTIR, Nicolet iS10, USA). The valence

states of manganese component were analyzed by X-ray photoelectron spectrometer (XPS, Thermo Fisher Scientific, USA). The metal ion contents in nanoparticles were determined by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer, USA, Agilent 7800). The optical absorption profile in the UV-vis region was acquired using an UV-vis spectrophotometer (Shimadzu Company, Japan). All electron spin resonance (ESR) spectra measurements were acquired by an ESR spectrometer (Bruker A300-10/12, Germany) at ambient temperature.

Magnetic resonance imaging

To verify the MR contrast capacity of RMnMels, relaxivity measurement was carried on a MR apparatus (Bio-Spec, Bruker, Karlsruhe, Germany). RMnMels with various metal molar concentrations (0-1 mM) were dispersed in ultrapure water. Phantom images were acquired at room temperature and the imaging parameters were set as follows: (i) T_1 RARE sequence: repetition time (TR)/echo time (TE): 1500/8 ms; slice thickness: 1 mm; field of view: 2.5×2.5 cm; matrix: 256×256 ; (ii) T_1 -map sequence: TR in the range from 447 ms to 5500 ms; TE: 8 ms; slice thickness: 1 mm; field of view: 2.5×2.5 cm; matrix: 256×256 . The r_1 relaxivity was determined by a linear fitting function between $1/\text{relaxation time (s}^{-1}\text{)}$ and manganese ion molar concentrations (mM). *In vivo* T_1 -weighted MR images were acquired on a MR apparatus (M3TM, Aspect Imaging, Israel). The parameters were set as follows: TR/TE: 500/12 ms; slice thickness: 0.8 mm; field of view: 3.0×6.0 cm; matrix: 256×128 . During image acquisition, all mice were anesthetized using a mixture of isoflurane and compressed air.

Photoacoustic imaging

3 mL RMnMels with various concentrations (0-500 $\mu\text{g/mL}$) were placed in a cylindrical model for photoacoustic signal detection, using a multispectral photoacoustic tomography imaging system (MSOT, iTheraMedical, Germany). The parameters were set up to allow absorbance of 690-900 nm. The photoacoustic signal intensity was quantitatively analyzed.

Biodistribution of RMnMels

200 μL RMnMels solution (12 mg/kg body weight) was intravenously injected into PC3 tumor bearing mice. T_1 -weighted MR images were acquired prior to and at various time points post injection of RMnMels using a 1.5 T MR scanner with aforementioned imaging parameters. To quantify the contrast enhancement, the signal intensity was determined by finely analyzing ROI of the images. To eliminate possible variations among mice, normalized T_1 relaxation times were analyzed against post-injection time and by setting that of prior to injection as 1.0. The photoacoustic signals in the tumor regions were acquired prior to and at various time points post injection of RMnMels using MSOT system, with images acquired in 8 min with 0.5 mm per step.

Photothermal conversion property

To measure the photothermal conversion property of RMnMels, 200 μ L RMnMels solution with various concentrations (0-200 μ g/mL) underwent exposure to a 690 nm laser (500 mW/cm², 300 s). And 200 μ g/mL RMnMels aqueous solution were exposed to a 690 nm laser at various laser power densities for 300 s. The infrared thermal images were acquired and the temperature variations were monitored using an infrared thermal camera (FLIR E75-42 Advanced Thermal Imaging Camera, USA).

Multi-antioxidative activities of RMnMels

For DPPH scavenging assay, 5 mg DPPH solution in 25 mL ethanol (200 μ g/mL) was added to various concentrations of RMnMels (0-100 μ g/mL) solution with 1:1 volume ratio. After kept 30 min away from light at room temperature, the optical absorbance at 516 nm was determined. The spin-trap free radical scavenging DEPMPO was used as the trapper for O₂^{•-} and •OH radicals. The stock solution of O₂^{•-} radicals was produced at room temperature. DMSO containing saturated air was fully mixed with 5 mmol/L NaOH solution and 1% H₂O. After fully stirred and placed for 30 min, and then PBS solution containing EDTA (0.2 mM) and DMPO (40 mM) was added. ESR test was carried out at 77K. For electron paramagnetic resonance (EPR) measurements, the DMSO solution was added into a water solution containing EDTA (0.2 mM), DEPMPO (40 mM), and PBS/PMnMels/RMnMels/SOD, and the EPR spectra were recorded. •OH was measured using an EPR spectra spin-trapping method with DMPO-OH as the spin-trapping agent in the condition of absence or presence of nanoparticles. The •OH was produced by a Fenton reaction between Fe²⁺ ions and H₂O₂. Then, nanoparticles were added into the mixture of DMPO and H₂O₂, followed by Fe²⁺ ions addition. Last, RMnMels were pre-incubated with DMPO and Fe²⁺, followed by H₂O₂ addition.

The effect of RMnMels on mitochondrial function

The effect of RMnMels on mitochondrial function was measured by Seahorse XFp Extracellular Flux Analyzer (Agilent Technologies, USA). All experiments were performed according to the manufacturer's protocol. Seahorse XFp Cell Mito Stress Test Kit (Agilent Technologies) were used to measure oxygen consumption rate (OCR), basal respiration (Basal Res), and spare respiratory capacity (Spare Res Cap). Briefly, 2×10⁴ PC3 cells were plated per well and seeded in a Seahorse XFp cell culture microplates and incubated for 12 h at 37°C, respectively. 50 μ g/mL RMnMels were added and co-incubated with cells for another 24 h. Untreated cells served as negative control. The results were normalized to cell number. Data were analyzed using Seahorse XFp Wave software.

Reactive Oxygen Species (ROS) levels *in vitro*

RAW 264.7 cells were first pre-stimulated with PBS, 100 ng/mL LPS and 50 μ g/mL RMnMels for 24 h, respectively. Then, cellular ROS levels were detected using a DCFDA-Cellular ROS Detection Assay Kit (Solarbio, Beijing, China). According to the instructions of the manufacturer, 10 μ M 2',7'-dichlorofluorescein diacetate (DCFDA) was added at 37°C for 30 min, and fluorescence was measured using a confocal laser scanning microscopy (Andor Dragonfly, UK).

Cell experiments

Human prostate cancer cell PC3 and RAW 264.7 cells were obtained from the Cell Culture Collection of Chinese Academy of Sciences (Shanghai, China). PC3 and RAW 264.7 cells were maintained in MEM and DMEM containing 10% FBS and 1% penicillin/streptomycin, respectively. The cells were kept in a homothermal cell incubator containing 5% CO₂ at 37°C.

For cytotoxicity assay

PC3 and RAW 264.7 cells were seeded in 96-well cell culture plates (1×10⁴ cells/well) and incubated overnight at 37°C, respectively. RMnMels with various concentrations were added and co-incubated with cells for another 24 h. A standard CCK8 assay was carried out to assay the cell viability. For cellular uptake experiment, PC3 cells were co-incubated with 50 µg/mL RMnMels for 12 hours. Then, cells were washed for several times and optical microscope images were acquired. RAW 264.7 cells were co-incubated with various concentrations of RMnMels for 12 hours, then visualized by an optical microscope. For bio-TEM, cells were harvested by trypsin digestion and centrifugation, fixed in 2.5% cold glutaraldehyde, dehydrated, embedded, sectioned, and stained for TEM observation.

Cellular uptake and degradability assay

To evaluate the degradation profiles *in vitro*, 100 µg/mL RMnMels were suspended into PBS (pH=7.4) without or with H₂O₂ (2.5 mM), respectively. The solution was kept stand at room temperature. At selected time intervals, digital photos of the solutions were taken, and then 100 µL solution was collected for detecting the accumulated degradation content using an UV-vis spectrometer. TEM images were obtained for intuitive observation of time-dependent structural evolution during degradation process. For *in vitro* photothermal cytotoxicity, PC3 cells were seeded in a 12-well cell culture plate (5×10⁴ cells/well) and incubated overnight at 37°C. The cells were co-incubated with 100 µg/mL PMnMels and RMnMels for 12 hours, respectively. Then, cells were washed for several times and replaced with fresh medium, following by exposure to a 690 nm laser at 500 mW/cm² for 5 min. After incubation for another 4 hours, Calcein AM and PI co-staining was performed for 30 min, and then fluorescence images were acquired. To quantify the photothermal cytotoxicity of RMnMels, PC3 cells were seeded in a 96-well cell culture plate (1×10⁴ cells/well), and co-incubated with various concentrations of RMnMels for 12 hours. Then, cells were exposed to a 690 nm laser at 500 mW/cm² for 5 min, washed for several times and then incubated in fresh cell culture media for another 4 hours. A standard CCK8 assay was carried out to assay the cell viability.

The effect of RMnMels on M2 macrophage polarization *in vitro*

RAW264.7 cells were first pre-stimulated with 100 ng/mL IL-4 for 24 hours to obtain M2-like phenotype, and then co-incubated with or without 10 µg/mL RMnMels for another 24 hours. The supernatants were carefully harvested for measuring TNF-α, IL-6, IL-1β, and IL-10 levels using ELISA assay kits (R&D Systems, USA). Meanwhile, the cells were collected for determining the gene expression levels of CD86,

TNF- α , iNOS, Arginase-1 and CD206 by reverse transcription-polymerase chain reaction (RT-PCR). The primer sequences used are shown in Table S1. The gene expression level were normalized to the housekeeping gene reduced glyceraldehyde-phosphate dehydrogenase (GAPDH). For determining the protein expression levels of iNOS, IL-1 β , COX-2 and TNF- α , western blot assay was carried out, with β -actin as the internal reference. To detect macrophage surface markers, cells were labelled with CD86-PE and CD206-APC mouse monoclonal antibodies. For each sample, at least 3×10^4 cells were run on a flow cytometer and data was analyzed using FlowJo software (BD Bio-sciences, USA).

Flow cytometry assay of cells in tumor tissues

Tumor tissues were collected and first digested into single-cell suspension at 37°C, and then filtered with a 70 μ m cell screen. Cells were collected centrifugally and dispersed in PBS containing 1% FBS. Then, cells were stained with 1 μ L Fixable Viability Stain 510 (*FVS510*) (BD Horizon™) for 15 min in 1 mL PBS at 4 °C to distinguish live and dead cells. Cells were then washed and stained with 0.5 μ g anti-mouse CD16/32 for 10 min at 4°C. Then, the cells were stained with APC-Cy7-labelled anti-mouse CD45, APC-Cy5.5-labelled anti-mouse CD11b, Alexa Fluor 488-labelled anti-mouse F4/80, APC-labelled anti-mouse CD163, PE-labelled anti-mouse CD86, respectively (BioLegend). Cells were then washed three times before flow cytometry analysis. The proportion of cells and the fluorescence intensity of TAMs were measured by flow cytometry, using an isotype-matched control antibody for background subtraction. Samples were further analyzed on BD LSR II instrument (BD Biosciences, USA).

Animal experiments

BALB/c nude mice (male, 6-8 weeks, 18-22 g) were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd., Beijing, China. All the animal experiments were approved by the Animal Care and Use Committee of Chinese Academy of Sciences, Beijing, China. Tumor model was constructed by subcutaneously inoculating 1×10^6 PC3 cells suspension in 150 μ L cell culture medium into the left back of mice. The mice were used for further experiments when the tumor volumes reached about 100 mm³, which was about three weeks after tumor inoculation.

To investigate the impact of RMnMels-mediated macrophage polarization and hyperthermia therapy on tumor growth, the PC3 tumor-bearing mice were randomly assigned to five groups: PBS (group I), PBS plus 690 nm laser irradiation (group II), RMnMels only (group III), PMnMels plus 690 nm laser irradiation (group IV), and RMnMels plus 690 nm laser irradiation (group V). The mice in group III, IV and V were intravenously injected with nanocomposites with 12 mg/kg BW. At 6 h post injection, the tumor sites were irradiated by a 690 nm laser at 500 mW/cm² for 15 min. Thermal images at the tumor sites were obtained using a FLIR E75-42 Advanced Thermal Imaging Camera. The tumor sizes were measured every two days and the tumor volumes were calculated as volume = (length $\times$ width $\times$ width)/2. The body weight and tumor growth profiles were monitored for a period of 19 days post treatment, and then the mice were euthanized and their tumor tissues were collected for further analysis.

Serum biochemical measurement

Blood was collected from tumor-bearing mice on day 19 post treatment and healthy mice at different time points (12 mg/kg BW). Mice that received PBS treatment were set as control. To obtain serum, fresh ocular vein blood samples were harvested from mice, then centrifuged for three times at a speed of 3500 rpm for 15 min. The serum biochemical indicators were measured using an automatic biochemical analyzer (Cobas 8000, Roche, Germany).

Histological analysis

Hematoxylin and eosin (H&E) staining, TUNEL and Ki-67 immunohistochemical staining were performed. The mice were euthanized on the 19th day after different treatments, and their main organs were harvested and fixed in 4% formalin overnight. The samples were embedded and sectioned at a thickness of 5 µm. The sections were stained and images were taken using 3D HISTECH Pannoramic 250 (3DHISTECH, Hungary).

Declarations

Supplementary Information

Supplementary Information is available from the Online Library or from the author.

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Authors' contributions

Y. Liu, H. Liu,: investigation, data curation, writing—original draft, funding acquisition. W. Shang, H. Hui, J Wu, W. Zhang, P. Gao, K. Guo, writing—review and editing. Y. Guo, J. Tian conceptualization, supervision, funding acquisition, writing— review and editing. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

All the animal experiments were approved by the Animal Care and Use Committee of Chinese Academy of Sciences, Beijing, China.

Consent for publication

All authors agree to be published.

Data Availability Statement

All data used to generate these results is available in the main text and Supplementary Information.

Competing interests

The authors declare no conflict of interest.

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Schema

Schema is available in supplementary section

Figures

Figure 1

Characterization of MnMels and imaging performance of RMnMels. (a) SEM image of MnMels. Scale bar, 500 nm. (b) TEM image of MnMels. Scale bar, 500 nm. (c) EDX analysis. (d) Elemental mapping images. Scale bar, 500 nm. (e) High-resolution Mn 2p_{1/2} and Mn 2p_{3/2} XPS spectra. (f) FTIR spectra. Photoacoustic/MR imaging evaluation. (g) In vivo T1-weighted MR images of PC3 tumor-bearing mice prior to and at various time points post injection of nanoparticles. (h) The linear relationship for the r1

a UV-Vis spectra of RMnMels (black), PMnMels (red), and MnMels (blue) in water. The x-axis is Wavelength (nm) from 300 to 1000, and the y-axis is Absorbance (a.u.) from 0.0 to 2.5.

b Photothermal heating curves of RMnMels at different concentrations (0, 12.5, 25, 50, 100, 250, 500 $\mu\text{g/mL}$). The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is Absorbance at 690 nm. A linear fit is shown with $y=0.0033X+0.06$ and $R^2=0.9983$.

c Photodynamic heating curves of RMnMels at different laser powers (300, 350, 400, 450, 500 mW/cm^2). The x-axis is Time (s) and the y-axis is Temperature ($^{\circ}\text{C}$).

d Photodynamic heating curves of RMnMels at different concentrations (0, 25, 50, 100, 200 $\mu\text{g/mL}$). The x-axis is Time (s) and the y-axis is Temperature ($^{\circ}\text{C}$).

e Bar graph showing normalized absorbance (%) of RMnMels at different concentrations (100, 50, 25, 12.5, 6.25, 0 $\mu\text{g/mL}$).

f EPR spectra of RMnMels and SOD. The x-axis is Magnetic field (G) from 3460 to 3560, and the y-axis is EPR amplitude.

g EPR spectra of RMnMels and SOD. The x-axis is Magnetic field (G) from 3450 to 3570, and the y-axis is EPR amplitude.

h Bar graph showing cell viability (%) of RMnMels at different concentrations (0, 12.5, 25, 50, 100 $\mu\text{g/mL}$).

i Bar graph showing cell viability (%) of RMnMels at different concentrations (0, 12.5, 25, 50, 100 $\mu\text{g/mL}$).

j Fluorescence microscopy images of cells treated with PBS, RMnMels, and RMnMels + Laser. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

k Bar graph showing cell viability (%) of RMnMels at different concentrations (0, 12.5, 25, 50, 100 $\mu\text{g/mL}$).

l Bar graph showing cell viability (%) of RMnMels at different concentrations (0, 12.5, 25, 50, 100 $\mu\text{g/mL}$).

m Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

n Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

o Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

p Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

q Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

r Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

s Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

t Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

u Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

v Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

w Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

x Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

y Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

z Fluorescence microscopy images of cells treated with PBS, LPS, and RMnMels. The x-axis is Concentration ($\mu\text{g/mL}$) and the y-axis is OCR (pmol/min/10000 Cells).

Photothermal properties, optical absorption and free radical scavenging properties of nanoparticles. (a) UV-vis spectra. (b) The linear relationship between the optical absorbance at 690 nm and RMnMels mass concentrations. (c) Photothermal heating curves of 200 $\mu\text{g/mL}$ RMnMels during 690 nm laser irradiation with various laser densities. (d) Photothermal heating curves of RMnMels with various concentrations during 690 nm laser irradiation at 500 mW/cm^2 . DPPH (e), $\text{O}_2^{\cdot-}$ (f), and $\cdot\text{OH}$ (g) scavenging ability of nanoparticles. (h) Effects of RMnMels on oxygen consumption rate of PC3 cells. (i) Quantification of basal respiration and spare respiratory capacity. (j) Calcein-AM/PI co-stained images of PC3 cells after different treatments. Scale bar, 20 μm . (k) PC3 cells viability after different treatments. (l) ROS fluorescence intensity in PC3 cells after different treatments. (m) Representative confocal images of ROS staining. (n) Fluorescence images for intracellular ROS in PC3 tumor tissue slices after RMnMels plus laser treatment. Scale bar, 50 μm . All experiments were run at least in triplicate. The statistical differences were quantified using Student's t test (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

Figure 3

RMnMels for M2-to-M1 macrophages repolarization. (a) RT-PCR analysis of mRNA expression levels in RAW 264.7 cells. (b) Western blot analysis of protein expression levels in RAW 264.7 cells. (c) Flow cytometric assay of RAW 264.7 cells polarization toward M1 phenotype. ELISA assay for the levels of cytokines in RAW 264.7 cells (d) and PC3 tumor tissues (e) after different treatments. (f) Representative immunofluorescence images showing the phenotypes of macrophages after different treatments. Scale bars, 20 μ m. (g) Representative immunofluorescence images of CD163 and iNOS in PC3 tumor tissue slices after different treatments. Scale bars, 20 μ m. All experiments were run at least in triplicate. The statistical differences were quantified using Student's t test (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

Figure 4

In vivo therapeutic evaluation of nanoparticles in PC3 tumor-bearing mice. (a) Schematic illustration of the time schedule for therapeutic treatment. Infrared thermal images (b) and temperature evolution curves (c) at tumor sites during different treatments. (d) Tumor growth on day 19 after different treatments. (e) The percentages of TAMs (CD45+CD11b+F4/80+) using flow cytometry. (f) The percentage of M1-like phenotype (CD86^{high} in CD45+CD11b+F4/80+ cells) and M2-like phenotype

(CD163^{high} in CD45+CD11b+F4/80+) using flow cytometry. (g) Quantification of TAMs, M1-like TAMs, and M2-like TAMs and the ratio of M1/M2 TAMs infiltrated in tumor tissues by flow cytometry (n= 3). Data were presented as mean±SD. The statistical differences were quantified using Student's t test (ns, no significance, * p < 0.05, ** p < 0.001, *** p < 0.0001).

Figure 5

Histological analysis and safety evaluation. Representative H&E stained images of liver tissue slices (a) and inguinal lymph nodes (b) on day 19 post treatment. Scale bars, 500 μm. (c) Quantification of hepatic metastatic nodules and tumor ipsilateral inguinal lymphatic metastatic nodules on day 19 post treatment. (d) Digital photographs of liver, tumor ipsilateral inguinal lymph nodes and tumor tissues in PC3 tumor-bearing mice on day 19 post treatment. Scale bars, 10 mm. (e) H&E, TUNEL and Ki67 stained

images of tumor tissue slices on day 19 post treatment. Scale bar, 100 μm . (f) Serum biochemical indexes of mice on day 19 post treatment.

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