Original Research Paper

**pH-activatable oxidative stress amplifying dissolving microneedles for combined chemo-photodynamic therapy of melanoma**

Yanjuan Huang a,1, Hualu Lai b,1, Jingwen Jiang a,1, Xiaoyu Xu a, Zishan Zeng a, Lingling Ren a, Qiuxing Liu b, Meixu Chen a, Tao Zhang a, Xin Ding b, Chunshun Zhao a, Shengmiao Cui b,c,*

a School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, China
b School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou 510006, China
c Guangdong Provincial Key Laboratory of Advanced Drug Delivery Systems, Guangdong Pharmaceutical University, Guangzhou 510006, China

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**A B S T R A C T**

Photodynamic therapy (PDT)-mediated oxidation treatment is extremely attractive for skin melanoma ablation, but the strong hydrophobicity and poor tumor selectivity of photosensitizers, as well as the oxygen-consuming properties of PDT, leading to unsatisfactory therapeutic outcomes. Herein, a tumor acidic microenvironment activatable dissolving microneedle (DHA@HPFe-MN) was developed to realize controlled drug release and excellent chemo-photodynamic therapy of melanoma via oxidative stress amplification. The versatile DHA@HPFe-MN was fabricated by crosslinking a self-synthesized protoporphyrin (PpIX)-ADH-hyaluronic acid (HA) conjugate HA-ADH-PpIX with “iron reservoir” PA-Fe 3+ complex in the needle tip via acylhydrazone bond formation, and dihydroartemisinin (DHA) was concurrently loaded in the hydrogel network. HA-ADH-PpIX with improved water solubility averted undesired aggregation of PpIX to ensure enhanced PDT effect. DHA@HPFe-MN with sharp needle tip, efficient drug loading and excellent mechanical strength could efficiently inserted into skin and reach the melanoma sites, where the acidic pH triggered the degradation of microneedles, enabling Fe-activated and DHA-mediated oxidation treatment, as evidenced by abundant reactive oxygen species (ROS) generation. Moreover, under light irradiation, a combined chemo-photodynamic therapeutic effect was achieved with amplified ROS generation. Importantly, the Fe-catalyzed ROS production of DHA was oxygen-independent, which work in synergy with the oxygen-dependent PDT to effectively destroy tumor cells. This versatile microneedles...
1. Introduction

Melanoma is one of the malignant, most aggressive and most dreadful skin cancer because of its high mortality, treatment-resistant and high metastatic potential [1–3]. Although it only occupies for 4% of skin cancers, melanoma is the primary reason of skin cancer mortality, posing huge threat to human health [4,5]. Surgical excision is the first choice for early-stage melanoma, but it can easily cause large skin defects and incompletely removed tumor tissues have a high risk of recurrence [6,7]. Chemotherapy, the primary treatment choice for melanoma, has been severely hindered by unsatisfactory efficacy and inevitable side effects, leading to poor quality of life [2]. Therefore, there is an urgent need to exploit innovative treatment strategies/systems that are effective, safe and economical for melanoma patients.

Reactive oxygen species (ROS)-mediated tumor oxidative therapy that utilizes exogenous ROS to aggravate intracellular oxidative stress for redox homeostasis disruption, has emerged as a promising tumor-specific treatment strategy [8]. ROS, primarily including singlet oxygen (¹O₂), hydroxyl radical (·OH), hydrogen peroxide (H₂O₂) and superoxide radical (O₂⁻·), is a vital signal regulating molecule in normal physiological conditions [9]. According to reports, tumor cells soaked in intrinsic oxidative stress are more susceptible to additional oxidative damages than normal cells [10], which highlights the advantages of ROS-mediated oxidation treatment. Among various oxidation therapies, photodynamic therapy (PDT) is extremely attractive for melanoma treatment owing to its high efficiency, non-invasiveness, spatiotemporal controllability and convenient for superficial administration [11–14]. PDT can generate highly-cytotoxic ¹O₂ by using light, photosensitizers and oxygen to exterminate cancer cells and tissues, showing great potential in clinical application [15–18]. However, the efficacy of oxygen-dependent PDT can be compromised in the tumor hypoxic region, so combining other oxygen-independent ROS inducers with PDT can be an inspirational strategy to effectively destroy tumor cells. Fortunately, dihydroartemisinin (DHA), a extensively used first-line antimalarial drug [19], has exhibited potential therapeutic effect in cancer therapy via the mechanism of inducing oxidative stress [20–22]. Interestingly, Fe(II) has proved to be an effective catalyst, which can catalyze the breakage of endoperoxide bridges of DHA to generate abundant ROS independent of oxygen, amplifying DHA-mediated intracellular oxidative levels [23,24]. Therefore, integrating PDT with Fe-augmented DHA-mediated oxidative therapy can be an effective strategy to enhance tumor suppression.

However, systemic administration of photosensitizers and other free drugs for combined oxidative therapy is severely hindered by poor water solubility [14], low tumor selectivity [25], and asynchronous distribution of photosensitizers or anti-cancer drugs in tumor tissues. In the last decades, encapsulation of drugs into nano-based drug delivery systems (NDDSs) has been extensively studied and has shown great potential in tumor treatment [26–32]. Owing to the unique biophysicochemical properties, such as improved water solubility and pharmacokinetics behavior, as well as enabling tumor-specific accumulation and controlled release of various co-delivered agents, NDDSs can achieve higher therapeutic efficacy with minimal toxicity [27,33–37]. Although promising, NDDSs that usually administered intravenously still face several limitations in vivo application. For example, uncontrolled drug leakage, inadequate drug loading, ineluctable self-quenching effect of hydrophobic PSs, inevitable systemic side effects and poor patient compliance [38]. Importantly, it is documented that only a small fraction of intravenously administered nanodrugs (0.7%, median) truly reach the tumor tissues after facing various physiological barriers, resulting in poor tumor accumulation and undesirable side effects to healthy organs [39].

To address these challenges and considering the biological characteristics of skin cancer, dissolving microneedles (DMNs) have recently emerged as an efficient, painless and non-invasive alternative to efficiently deliver anticancer drugs to localized lesions, showing great potential for skin melanoma [4,40–43]. DMNs, a new generation of transdermal drug delivery platform, can insert into stratum corneum and create an array of microchannels to directly deposit therapeutic agents on the tumor site, and subsequently provide uniform three-dimensional drug distribution, avoiding off-target effects to minimize toxicity to healthy organs [40,44–46]. Although DMNs has achieved great success in delivering various drugs for treating different disease in recent years, DMNs with controlled drug release functions are still in great demand.

Herein, we developed an ingenious tumor acidic microenvironment activatable DMNs (DHA@HPFe-MN) for realizing pH-triggered drug release and localized synergistic chemo-photodynamic therapy of melanoma via oxidative stress amplification. Primarily, hyaluronic acid (HA), an abundant biocompatible and biodegradable natural polysaccharide in skin, was covalently linked with adipic dihydrazide (ADH) to synthesize HA-ADH conjugate. Then, protoporphyrin IX (PpIX), a clinically common used hydrophobic photosensitizer, was linked to HA-ADH to synthesize HA-ADH-PpIX conjugate with good water solubility, intending to avert unexpected aggregation of PpIX for efficient PDT. Subsequently, an “iron reservoir” named “PA-Fe³⁺ complex” was fabricated by coordinating the catechol hydroxyl group of protocatechualdehyde (PA) with Fe³⁺, which was then served as aldehyde containing...
crosslinker. The photosensitizer-carrying microneedle matrix material HA-ADH-PpIX, and the PA-Fe\(^{3+}\) crosslinker with dihydroartemisinin (DHA) added were poured into the female mode and concentrated to the tip of the microneedles. Then the pH-responsive hydrogel DMN (DHA@HPFe-MN) co-delivering photosensitizer PpIX, Fe\(^{3+}\) and DHA was prepared by the formation of pH-responsive dynamic covalent acylhydrazone bond (Scheme 1A).

After punctured into the tumor tissue in skin, the microneedles were hydrated in the tissue fluid and degraded in the acidic tumor environment to release DHA and PA-Fe\(^{3+}\) complex (Scheme 1B). After internalized by tumor cells, the PA-Fe\(^{3+}\) complex was reduced by high levels of glutathione (GSH) to release Fe\(^{2+}\). Under the catalysis of Fe\(^{2+}\), the endoperoxide bridge of DHA was destroyed, resulting in ROS generation to kill tumor cells. Simultaneously, under a 633 nm light irradiation, PpIX could produce ROS for localized PDT, further increasing the ROS levels in tumor cells to amplify intracellular oxidative stress, achieving a combined chemo-photodynamic treatment effect. Noteworthy, the ROS generated by DHA under the Fe\(^{3+}\) catalyst did not require oxygen participation. Therefore, the designed drug delivery system can overcome the limitation of oxygen-dependent PDT in tumor hypoxic regions. These findings revealed that the designed pH-responsive DMNs can serve as an attractive topical delivery system for combined chemo-photodynamic therapy of melanoma with minimized systemic toxicity.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (HA, M\(_{w}\) = 3–10 kDa and 200–400 kDa) was obtained from Bloomage Freda Biopharm Co., Ltd. (Shandong, China). Adipic dihydrazide (ADH), 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC·HCl), \(\cdot\)n-hydroxy succinimide (NHS), 4-dimethylaminopyridine (DMAP), 9,10-anthracenediyli-bis(methylene)dimalonic acid (ABDA), dihydroartemisinin (DHA), coumarin 6 (C6), rhodamine B (RhB), 1,3-diphenylisobenzofuran (DPBF) and fluorescein diacetate (FDA) were obtained from Aladdin Reagents Co., Ltd. (Shanghai, China). Protoporphyrin IX (PpIX) was purchased from Howei Pharm (Guangzhou, China). Iron (III) chloride hexahydrate (FeCl\(_3\)·6H\(_2\)O, >99.0\%) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ferrous sulfate was purchased from Zhiyuan Reagent Co., Ltd. (Tianjing, China). Protocatechualdehyde (PA) and 1,10-phenanthroline monohydrate were obtained from Bide.
Pharmatech Co., Ltd. (Shanghai, China). Glutathione (GSH) was obtained from Energy Chemical Co., Ltd. (Shanghai, China). 3-(4,5-dimethyl-2-thiazolyl)–2,5-diphenyl tetrazolium bromide (MTT), cobaltous chloride (CoCl₂·6H₂O), 2,7’-Dichlorodihydrofluorescein diacetate (DCFH-DA) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, USA). Iron ion (Fe³⁺) probe was purchased from Heliosense Biotechnology Co., Ltd. (Xiamen, China).

**Cell lines** B16 melanoma cell lines were purchased from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China) and cultured in RPMI-1640 medium (Gibico) containing 10% FBS (Gibico) and 1% penicillin–streptomycin (100 U/ml). The cells were grown in a humidified incubator in the presence of 5% CO₂ and 95% air at 37 °C.

**Animals** Healthy C57BL/6 female mice (5–6 weeks, 17–18 g) and male SD rats (180 ± 20 g) were obtained from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China). All animal experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

### 2.2. Synthesis and characterization of microneedle matrix materials

**2.2.1. Synthesis and characterization of HA-ADH**

HA-ADH was synthesized based on our previously published method [47]. HA (Mw=3–10 kDa, 1 g) and ADH (3 g) were dissolved in 100 ml distilled water and stirred at room temperature. After the two compounds were completely dissolved, the pH of the solution was adjusted to 4.75 by 0.1 M HCl. Then, EDC was added in an equimolar amount of HA to activate the reaction. The reaction was stirred at room temperature overnight, and the pH was continuously monitored and maintained at 4.75 by the addition of 0.1 M HCl. Then, 0.1 M NaOH solution was added to adjust the pH to neutral, and the reaction was terminated. The resulting mixture was placed into a dialysis bag (MWCO = 1000 Da) and dialyzed against distilled water for 48 h. HA-ADH was obtained by lyophilization and characterized by ¹H NMR (400 MHz, AVANCE-400, Bruker, Germany) and Fourier transform infrared (FTIR) spectroscopy (VERTEX 70, Bruker, Germany).

**2.2.2. Synthesis and characterization of microneedle matrix material HA-ADH-PpIX**

To synthesize HA-ADH-PpIX, briefly, PpIX (60 mg) was dissolved in 30 ml DMSO, then DMAP (72 mg) and NHS (72 mg) were dissolved in 5 ml DMSO and added into the PpIX solution. The reaction solution was stirred for 30 min at room temperature, then it was added dropwise into a pre-prepared 100 ml 1% HA-ADH aqueous solution and stirred at room temperature in the dark. The pH of the reaction system was continuously monitored and maintained at 7.0. After 48 h of reaction, the solution was successively dialyzed (MWCO = 1000 Da) against a mixed solution containing pure water and ethanol (4:1, v/v) and ultra-pure water with pH 7.0 adjusted by ammonia water, and the dialysis was carried out in dark for 72 h. After that, the supernatant was collected by centrifugation (10 000 g, 10 min) to eliminate the undissolved by-products, and it was then freeze-dried to harvest HA-ADH-PpIX. The final product was characterized by UV-vis spectroscopy (UV2600, Techcomp, China) in a mixed solution of DMSO:H₂O (1:1, v/v). The content of PpIX in HA-ADH-PpIX was detected by a UV–vis spectroscopy at 403 nm.

2.2.3. Synthesis and characterization of PA-Fe³⁺ coordination complex

PA (100 mg) was dissolved in ethanol, then FeCl₂·6H₂O solution (65 mg) was added dropwise with a molar ratio of PA:Fe³⁺ equal to 3:1, and then the pH of the mixed solution was adjusted to 8.5 with 0.5 M NaOH solution. After stirring at room temperature for 3 h, the solvent of the reaction mixture was removed in a rotary evaporator and the solute was redissolved in 4 ml ethanol to a final Fe³⁺ concentration of 60 mM. The obtained PA-Fe³⁺–polyphenol coordination complex was characterized by UV–vis spectroscopy and FTIR.

### 2.3. In vitro ROS generation of HA-ADH-PpIX and DHA with Fe ion

2.3.1. ROS generation of HA-ADH-PpIX under light irradiation

ABDA, a water soluble ROS indicator, was applied to evaluate the in vitro ROS generation of HA-ADH-PpIX under light illumination. Briefly, 1 ml ABDA solution (150 μM in PBS) was mixed with an equal volume of PpIX (0.5%) in DMSO or HA-ADH-PpIX in water at an equivalent PpIX concentration of 10 μg/ml. Blank ABDA solution was set as control. Then each solution was irradiated or not with a 633 nm laser (50 mW/cm²) for 0.5, 10, 15, 20, 25 and 30 min. After irradiation, the absorbance decrease of ABDA at 401 nm of each mixture was recorded to evaluate the ROS generation capacity of HA-ADH-PpIX. The absorbance at 0 min was regarded as A₀, and the absorbance at other determined time points were regarded as Aₜ. The relative amount of residual ABDA (%) was calculated as Aₜ/A₀.

2.3.2. ROS generation of DHA with Fe²⁺

The Fe²⁺-activated ROS generation of DHA was detected using DPBF as a ROS indicator. DPBF can effectively capture and react with ROS in organic solvent, resulting in a decrease of absorbance at 412 nm. Briefly, 1 ml DPBF solution (0.48 mg/ml in ethanol) was mixed with 2 ml DHA, DHA + Fe²⁺, DHA + Fe³⁺, Fe²⁺ and Fe³⁺ solutions (ethanol:pH 5.0 PBS=2:1, v/v). Blank DPBF solution was set as control. At predetermined time points of 0, 10, 30, 60, 90, 120 and 180 min, the absorbance of DPBF of each mixture was recorded using a UV–vis spectrophotometer, and the decrease of absorbance at 412 nm was used to evaluate ROS generation. The amount of remained DPBF (%) was calculated similar to that of ABDA.

2.3.3. PA-Fe³⁺ complex generate Fe²⁺ under different GSH concentrations

The generation of Fe²⁺ was determined using phenanthroline as an indicator. Briefly, 1 ml PA-Fe³⁺ aqueous solution (100 μM Fe³⁺) was reacted with equal volumes of different GSH aqueous solutions (12.5, 25, 50, 100 and 200 mM) for 12 h, respectively. After that, 1 ml phenanthroline ethanol solution (0.1%) was added to the above mixtures and incubated for
another 5 h. Finally, the UV–vis spectrum of each sample at 400–550 nm was recorded by UV–vis spectroscopy.

2.4. Preparation of HPFe-MN and drug loaded DHA@HPFe-MN microneedles

The polydimethylsiloxane (PDMS) master mold of microneedles (121 needles) used in this study was manufactured by the company. The HPFe-MN microneedles were fabricated through a two-step micromolding method [6]. Briefly, 250 μl 50% HA-ADH-PpIX aqueous solution and 100 μl diluted PA-Fe³⁺ ethanol solution were added into a 2 ml DP. The mixture was then vortexed to obtain a drug-free blank microneedle matrix solution, which was immediately filled into the PDMS female mold, followed by repeated centrifugation (2504 g, 15 min) for three times to compress the material into the mold cavities. Then, excess matrix solution was wiped out from the surface of the mold, and the obtained female mold was dried at room temperature in a dryer under dark for 1 h. After that, 15% HA (Mw = 200–400 kDa) aqueous solution was poured on the female mold under centrifugation (2504 g, 5 min) to get a robust backing substrate of the microneedle patch. Finally, the entire mold was placed in a dryer at room temperature under dark for 24 h, and then gently peeled off the mold to obtain the PpIX and PA-Fe³⁺ complex co-loaded hydrogel-based microneedles (HPFe-MN).

For the preparation of DHA loaded HPFe-MN microneedles (DHA@HPFe-MN), DHA powder was uniformly dispersed within the above PA-Fe³⁺ ethanol solution beforehand. Subsequently, 100 μl of the above DHA containing PA-Fe³⁺ ethanol solution was mixed with 250 μl 50% HA-ADH-PpIX aqueous solution in a 2 ml DP, and then vortexed for 5 min to obtain the drug-containing microneedle matrix solution. Then, DHA@HPFe-MN was prepared following the same procedures to that of HPFe-MN. Similarly, replacing the 50% HA-ADH-PpIX aqueous solution with 50% HA-ADH aqueous solution, as well as replacing DHA containing PA-Fe³⁺ ethanol solution with DHA ethanol solution in the above preparation steps of DHA@HPFe-MN, the control microneedles DHA@MN was prepared.

2.5. Characterization of microneedles

The morphology of the microneedles was characterized by digital microscope and scanning electron microscope (SEM) (MA10, Zeiss, Germany). To observe the distribution of drugs in microneedles, C6 and RhB were added into the matrix solution and backing solution, respectively, to prepare fluorescent microneedles. The distribution of C6 and RhB was then observed by confocal laser scanning microscopy (CLSM) (LSM 710, Zeiss, Germany) to verify the successful preparation of the microneedles. The content of DHA in DHA@HPFe-MN was determined by an UV–vis spectrophotometer. Briefly, DHA@HPFe-MN was immersed in an acid mixed solution (ethanol:pH 5.0 PBS=1:1, v/v) at room temperature for more than 24 h to ensure complete dissolution of the microneedles. The above solution was then treated with 0.2% NaOH and the mixture was left at 50 °C for 30 min, then the absorbance of the samples at 290 nm was measured by UV–vis spectrophotometer using the dissolved microneedle solution of HPFe-MN treated with the same method as a blank control. The content of DHA was calculated by comparing the absorbance with a standard curve of DHA treated using the same method. For PpiX content detection, the above dissolved solution of microneedles was treated with DMSO, then the content of PpiX was detected by fluorescence spectrometer (Fluoromax-4, HORIBA, USA, Ex = 400 nm, Em = 630 nm). For Fe³⁺ content detection, the microneedles were dissolved by aqua regia, heated and dried, and then redissolved by 4% HNO₃. The content of Fe was measured by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) (Optima 8300, PerkinElmer, USA).

2.6. The structure and drug release behavior of microneedles

The drug-free blank microneedle matrix solution was prepared according to Section 2.4. After gelation of the mixed solution, the hydrogel formed by microneedle matrix solution was photographed and freeze-dried for FTIR analysis. To investigate the pH-triggered degradation of hydrogel, pH 7.4 and pH 5.0 PBS were added to the gelled microneedle matrix solution respectively, the transformation from gel to solution was monitored by a camera. To evaluate the pH-triggered drug release behavior of the microneedles, the fluorescent dye C6 was loaded as a model drug for the convenience of detection. Briefly, C6@HPFe-MN was prepared similar to that of DHA@HPFe-MN. One microneedle with 88 needles was divided into four groups, each group containing 22 needles was added with 0.5 ml pH 5.0, pH 6.5, pH 7.4 and pH 10.0 PBS containing 5% SDS, respectively. At predetermined time points, each group was photographed under a lamp and 365 nm light to observe the color and fluorescence changes of the samples.

To investigate a quantitative map of the in vitro drug release, microneedles were placed into a dialysis bag (MWCO: 3500 Da) and immersed in 30 ml pH 7.4, pH 6.5 and pH 5.0 PBS, respectively. The drug release was performed in a 37 °C shaker with a shaking rate at 100 rpm. At predetermined time points, 3 ml release medium was taken out and an equal volume of the fresh PBS was added. The content of the released Fe was measured by a high resolution continuum source flame atomic absorption spectrometry (HR-CS-FAAS, ContrAA 800, Analytik-jena, Germany). The cumulative release rate of Fe was calculated using the following formula:

\[
\text{Release} = \frac{\sum_{i=1}^{n-1} (C_{\text{sample}(i-1)} \times 30)}{C_{\text{MN}} \times 3} \times 100\% + \frac{\sum_{i=1}^{n-1} (C_{\text{sample}(i-1)} \times 30)}{C_{\text{MN}} \times 3} \times 3
\]

Where, \(C_{\text{sample}(i)}\) represents the Fe concentration in the release medium; \(C_{\text{MN}}\) represents the original Fe concentration in DHA@HPFe-MN.

2.7. The mechanical strength, skin insertion and penetration efficiency of microneedles

The mechanical strength of microneedles was recorded by TA1 texture analyzer (LLOYD, Germany). Briefly, the tip of the microneedles was fixed on the measuring table of the
texture analyzer, the loading force was set to 3 N, the maximum force was set to 90 N, and the compression rate was set to 10 mm/min, then the change curve of pressure vs displacement was recorded. Meanwhile, the morphological changes of the microneedles after mechanical test was observed under a digital microscope.

To investigate the in vitro skin insertion capacity, SD rats were euthanized and the abdominal skin tissues were excised, then DHA@HPFe-MN microneedles were pierced into the shaved skin, kept for 10 min. The microneedles were removed, the insertion site was photographed with a camera and the porosity was calculated. Finally, to detect the insertion depth, the above skin was fixed in 4% paraformaldehyde solution for 24 h, frozen and sectioned, and then observed by an optical microscope. For in vivo skin penetration evaluation, the microneedles containing C6 as a model drug (C6@HPFe-MN) were inserted into the shaved back skin of mice and fixed with medical tape. After that, the mice were euthanized and the related skin were harvest, then the distribution of the microneedles into the skin was observed by CLSM.

To investigate the drug penetration of DHA@HPFe-MN in the tissues, C6@HPFe-MN was pierced into the shaved skin of SD rats and fixed with transparent medical tape. After treated for 2 h, the microneedles treated skin was harvested for frozen fluorescent section, and then photographed by CLSM.

To investigate the in vivo skin insertion and recovery performance, female C57BL/6 mice were anesthetized with 4% chloral hydrate, then the DHA@HPFe-MN microneedles were pierced into the skin, fixed by medical tape and kept for 10 min, then the microneedles were removed, and the recovery of the skin at the insertion site was recorded.

2.8. In vitro cellular uptake and cytotoxicity of microneedles

To investigate the cellular uptake of DHA@HPFe-MN microneedles, B16 cells were seeded on glass coverslips in 12-well plates (1 \times 10^5 cells/well). After cultured for 24 h, cells were incubated with DHA@HPFe-MN in pH 6.5 RPMI 1640 medium for 4 h and 8 h respectively. After that, the media was replaced with 100 μM of Fe^{3+} probe and incubated for 30 min under dark. Then, the cells were washed with PBS, fixed with 4% paraformaldehyde, stained with DAPI and directly visualized by CLSM (LSM 710, Zeiss, Germany).

Firstly, cell viability assay of B16 cells treated with DHA, PA-Fe^{3+} and DHA with PA-Fe^{3+} was investigated. Typically, B16 cells seeded on 96-well plates (5 \times 10^4 cells/well) were incubated with different concentrations of DHA, PA-Fe^{3+} and PA-Fe^{3+} (250 and 500 μM) with DHA (0.781, 1.563, 3.125, 6.25, 12.5, 25, 50, 100 and 200 μM) for 24 h prior to MTT assay, and the absorbance of each well at 490 nm was recorded by a microplate reader (ELX800, Bio-Tek, USA).

Next, cell viability assay of B16 cells treated with microneedles was conducted. Briefly, B16 cells were seeded on 24-well plates and incubated for 24 h. To obtain hypoxia conditions, cells were incubated with 100 μM CoCl\(_2\) additionally. Cells in normoxia and hypoxia conditions were treated with DHA@MN, HPFe-MN and DHA@HPFe-MN microneedles with backing removed, respectively. After 20 h of incubation, cells were irradiated with a 633 nm light (0.8 W/cm^2) or kept in dark for 10 min, followed by further incubation for 4 h before adding MTT, then the cell viability was measured by MTT assay.

The cytotoxicity of microneedles under normoxia or hypoxia conditions was further verified by live/dead cell staining assay. Briefly, B16 cells seeded in 6-well plates (2 \times 10^5 cells/well) were incubated with DHA@MN, HPFe-MN and DHA@HPFe-MN microneedles with backing removed respectively for 20 h. Then cells were exposed not to 633 nm laser (0.8 W/cm^2) for 10 min. At 4 h after irradiation, the treated B16 cells were co-stained by FDA and PI and then observed by an inverted fluorescent microscope (IX73, Olympus, Japan).

2.9. Intracellular ros generation of microneedles under normoxia or hypoxia conditions

B16 cells were seeded on glass coverslips in 12-well plates (0.5 \times 10^5 cells/well), where cells incubated directly for 24 h. To obtain hypoxia conditions, cells were incubated with 100 μM CoCl\(_2\) additionally. Cells in normoxia and hypoxia conditions were incubated with various microneedle formulations for 24 h, then the drug medium was displaced by DCFH-DA (10 μM) and incubated for 30 min. After washed three times with PBS, cells were irradiated or not with 633 nm light (0.8 W/cm^2) for 10 min, then fixed with 4% paraformaldehyde, stained with DAPI and observed by CLSM.

2.10. Cell apoptosis assay of B16 cells treated with microneedles

B16 cells seeded in 6-well plates (2 \times 10^5 cells/well) overnight were incubated with DHA@MN, HPFe-MN and DHA@HPFe-MN microneedles with backing removed respectively for 24 h, then cells were irradiated (633 nm, 0.8 W/cm^2) or not for 10 min. After that, cells were harvested and the proportion of apoptosis was determined by flow cytometry using Annexin V/PI Apoptosis Kit.

2.11. In vivo antitumor efficiency of microneedles

To establish the B16 tumor mouse model, C57BL/6 female mice (5–6 weeks) were subcutaneously injected with B16 cells (1 \times 10^6 cells in 100 μl PBS) in the right flank of back. The growth states and tumor sizes of mice after inoculation were observed daily. When the tumors reached about 100–150 mm\(^3\), the mice bearing tumors were randomly divided into seven groups (n = 5) including the following: (1) Saline, (2) Saline + light, (3) HPFe-MN, (4) DHA-MN, (5) DHA@HPFe-MN, (6) HPFe-MN + light and (7) DHA@HPFe-MN + light. Mice were anesthetized with chloral hydrate and their back hairs were shaved and depilated. Then, one piece of microneedle formulations was inserted into the skin surrounding the tumor site and fixed with transparent medical tape. Mice were treated by the microneedles every other day for a total of four treatments. At 12 h after microneedle insertion, groups with light received a 633 nm laser irradiation on tumor for 10 min. The tumor sizes and body weights were determined every two days throughout the experiment. Tumor volume was calculated using the equation: length × width\(^2\) / 2.
At the end of the experiment, all tumor bearing mice were sacrificed and the tumors were excised, photographed and weighed. The tumor tissues were fixed with 4% paraformaldehyde, embedded into paraffin and sectioned for hematoxylin-eosin (H&E) staining and TUNEL staining analysis. At the meanwhile, the major organs (hearts, livers, spleens, lungs and kidneys) were collected, fixed and sectioned for H&E staining to evaluate the systemic toxicity.

2.12. Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistical differences between groups were determined by ordinary one-way ANOVA using Graphpad Prism 8.3.0 (GraphPad software, CA, USA). Statistical significance was considered when a value of P < 0.05 (∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001).

3. Results and discussions

3.1. Synthesis and characterization of the microneedle matrix material

3.1.1. HA-ADH-PpIX conjugate

HA-ADH-PpIX conjugate was synthesized as a water soluble microneedle matrix material according to the procedure exhibited in Fig. S1. First, under acidic and EDC-catalyzed conditions, the carboxyl groups of HA were linked with the hydrazide bond of ADH through a carbodiimide reaction to prepare the water soluble HA-ADH. The successful synthesis of HA-ADH and its substitution degree were characterized by H NMR. The characteristic peak of the methyl (−CH3) proton signal of the acetamido moiety of HA was observed at 1.99 ppm (labeled as "a"), while the characteristic peaks marked as "b" (2.2 ppm) were attributed to the methylene proton signal of ADH (Fig. 1A). Compared the H NMR spectra with HA and ADH, HA-ADH have both the methyl characteristic peak (#, 1.9 ppm) of HA and the methylene characteristic peaks (a and b) of ADH. Meanwhile, peak b has been split to generate a new peak b' (2.3 ppm) due to hydrazide bond formation, indicating that ADH has been successfully grafted on HA with excess hydrazide group exposed (Fig. 1A). According to the integration ratio of −CH3 (#, 1.9 ppm) of HA and methylene protons of ADH, the ADH substitution degree on HA was estimated to be 69%, referring to a well-documented formula reported previously [48,49]. The structure of HA-ADH was further confirmed by FTIR (Fig. S2). Comparing the FTIR spectra of HA and ADH, HA-ADH displayed the characteristic peak of HA, and a new peak at 1646 cm−1, which was attributed to the formation of −CONH−NH2 bond after ADH was attached to HA. The result further confirmed the successful preparation of HA-ADH.

Next, HA-ADH-PpIX conjugate was further synthesized by reacting the excess hydrazide groups of HA-ADH with the carboxyl groups of PpIX which were activated as an active ester under the catalysis by NHS and DMAP. After purification and freeze-drying, water soluble red-brown HA-ADH-PpIX with good water solubility was successfully harvested, which is intended to avert aggregation induced quenching of PpIX in water solution. HA-ADH exhibited invisible UV–vis absorption peaks, and the UV–vis spectra of HA-ADH-PpIX in water solution was similar to the characteristic absorption peaks of PpIX in DMSO solution, indicating that HA-ADH-PpIX was successfully prepared (Fig. 1B). Based on the UV–vis absorbance at 403 nm, the content of PpIX in HA-ADH-PpIX was determined to be about 1.32%, suggesting that a larger number of hydrazine groups were reserved in HA-ADH-PpIX for subsequent microneedle preparation.

3.1.2. PA-Fe3+ polyphenol coordination complex

The PA-Fe3+ polyphenol coordination complex (stoichiometric ratio of PA:Fe3+ = 3:1) was prepared at pH ≈ 8.5. As shown in Fig. 1C, when pH > 8, a wine red solution with a characteristic absorption peak at 450 nm was observed, indicating the formation of tris-catecholato-Fe3+ complex. When the pH was adjusted to below 3, immediate color change from wine red to dark green was observed, and the UV–vis absorption peak shifted from 450 nm to 660 nm, suggesting the formation of mono-catecholato-Fe3+ complex. When pH adjusted to 3 < pH < 7, the color of the solution changed to purple-black with characteristic absorption peak at 570 nm, indicating the formation of bis-catecholato-Fe3+ complex. The above phenomenon was identical to the reported literatures [50], indicating the successful preparation of tris-catecholato-Fe3+ complex (PA-Fe3+). The structure of PA-Fe3+ complex was further verified by FTIR (Fig. 1D). After coordination with Fe3+, the stretching vibration of hydroxyl group in PA between 3000 and 3500 cm−1 was broadened, and the characteristic peak of catechol group in PA at 1441 cm−1 was divided into two peaks at 1480 cm−1 and 1429 cm−1. Meanwhile, the red shift of the benzene C=C stretching vibration peaks (1650 - 1652 cm−1 and 1590 - 1605 cm−1) indicated that the charge density of C=C bond was affected by the conjugation effect after the coordination bond was formed between PA and Fe3+. The above results indicated the successful synthesis of PA-Fe3+ complex.

3.2. ROS generation of HA-ADH-PpIX and DHA

3.2.1. HA-ADH-PpIX under light irradiation

PpIX is a photosensitizer which commonly used for PDT in clinical practice. The ROS generation capacity of PpIX is a guarantee for its efficient PDT. The ROS generation capacity of HA-ADH-PpIX in water solution was detected using ABDA as a ROS indicator. ABDA can specifically capture and react with 1O2 to form an internal oxygen bridge in aqueous solution, leading to a decrease in its characteristic absorption peak at 405 nm [15,47,51]. As shown in Fig. 2A, ABDA solution, ABDA solution + light and PpIX groups had no evident ROS generation. PpIX + light group exhibited a small amount of ROS generation during 30 min, which might be caused by the aggregation-induced self-quenching of hydrophobic PpIX in water solution even with DMSO added. By contrast, HA-ADH-PpIX group displayed a sharp drop of absorbance during 30 min with light irradiation, while remained nearly unchanged without light irradiation, indicating the strong 1O2 quenching of PpIX by DMSO.
generation capacity of HA-ADH-PpIX under irradiation. These results demonstrated that conjugating of PpIX to HA could improve the water solubility of PpIX to prevent undesired aggregation and enhance \( ^1 \text{O}_2 \) production efficacy in aqueous solution.

3.2.2. DHA activated by Fe\(^{2+}\)

It is reported that Fe\(^{2+}\) can break the endoperoxide bridge of DHA, resulting in the generation of abundant ROS [22]. Thus, the Fe-activated ROS generation of DHA was detected using DPBF as a ROS indicator. As exhibited in Fig. 2B, DHA, Fe\(^{2+}\), Fe\(^{3+}\) and DHA + Fe\(^{3+}\) groups exhibited no obvious absorbance change of DPBF solution within 3 h, suggesting that none of these groups generated ROS in vitro. In sharp contrast, DHA + Fe\(^{2+}\) group exhibited significant decrease of absorbance during 3 h incubation period, indicating that DHA can easily be activated by Fe\(^{2+}\) to generate ROS.

The generation of Fe\(^{2+}\) by PA-Fe\(^{3+}\) complex under the reduction by GSH was detected using phenanthroline as an indicator. Phenanthroline can react with Fe\(^{2+}\) to form a stable orange-red complex, which has a maximum absorption peak at 512 nm. As shown in Fig. 2C, as the GSH concentration increased from 6.25 mM to 100 mM, the absorbance at 512 nm increased accordingly, indicating that Fe\(^{2+}\) in solution increased with increasing GSH concentration. The result suggested that PA-Fe\(^{3+}\) could generate Fe\(^{2+}\) in the presence of GSH in a concentration dependent manner, which could
be served as a “Fe$^{3+}$ reservoir” for subsequent combination therapy with DHA.

3.3. Preparation and characterization of DHA@HPFe-MN microneedles

The microneedles were fabricated through a two-step casting method as illustrated in Fig. 3A. HA was chosen as the matrix component because it is an abundant natural biocompatible glycosaminoglycan in skin [40]. HA was firstly modified with ADH and PpiX to serve as microneedle matrix material and PDT agent. HA-ADH-PpiX and PA-Fe$^{3+}$-complex were mixed and the mixture was poured into the PDMS female mode and concentrated to the tip of the microneedles. Then, the microneedle matrixes in the needle tips were cross-linked to form a black three-dimensional gel through the aldime condensation reaction between aldehyde group in PA-Fe$^{3+}$ and hydrazine bond in HA-ADH-PpiX with the formation of the pH-responsive acylhydrazone bond. Afterwards, 15% HA (Mw = 200–400 kDa) aqueous solution was casted in PDMS female mold under centrifugation to form the base of the microneedles. The digital micrograph of the dissolving microneedles observed that, the needle tip cross-linked by HA-ADH-PpiX and PA-Fe$^{3+}$ coordination complex is black, and the base composed of HA (Mw = 200–400 kDa) is colorless and transparent (Fig. 3B). The microneedle patch consisted of 121 (11 × 11) pyramidal needles, and each microneedle was uniform in shape without broken (Fig. 3C). The SEM image exhibited that the quadrangular pyramid-shaped needles with smooth surface and sharp needle tip were uniformly distributed on the base substrate with a height of 760 μm of each needle (Fig. 3C–3E). The drug-loading content of DHA@HPFe-MN microneedle was about 4.7 μg Fe$^{3+}$, 11.9 μg DHA and 9.8 μg PpiX per patch, determined by ICP-AES, UV–vis spectrophotometer and fluorescence spectrometer, respectively.

To visualize the drug distribution in the microneedles, fluorescence-labeled dissolving microneedles were prepared. C6 and RhB were chosen as the model dye which were added into the matrix solution and backing solution, respectively. As displayed in CLSM image, C6 was primary localized in the tips of the microneedles which emit green fluorescence, while RhB was mainly distributed in the base of the microneedles which emit red fluorescence (Fig. 3F). The result indicated that, owing to the gelation of the microneedle matrix material, C6 could be fixed on the microneedles, which was quite conductive for complete drug delivery to avoid drug loss caused by the drug diffusion to the backing layer during insertion.

3.4. The structure and in vitro drug release of microneedles

The microneedles were prepared by crosslinking the aldehyde group of PA-Fe$^{3+}$ complex (Fe reservoir) and the hydrazide bond of HA-ADH-PpiX to form acylhydrazone bond, which was reported to be pH-sensitive [52]. The successful formation of gel network was verified by FTIR, the characteristic absorption peak at 1605 cm$^{-1}$ indicated the successful formation of acylhydrazone bond (–C=–N–) in HPFe-hydrogel network (Fig. 4A). The formation of hydrogel network after mixing PA-Fe$^{3+}$ complex with HA-ADH-PpiX was further evidenced in Fig. 4B. Meanwhile, the formed hydrogel possess pH sensitivity, after adding pH 5.0 or pH 7.4 PBS to the hydrogel and treated it for 12 h, the pH 5.0 PBS treated group showed a gel to solution transition, while the pH 7.4 treatment group remained unchanged (Fig. 4B). Considering the sensitivity and convenience of detection, the pH-responsive drug release behavior of microneedles was further verified using C6@HPFe-MN loaded fluorescent microneedles. One microneedle with 88 needles was assigned to four groups, then each group containing 22 needles was added with PBS at different pH values (pH 5.0, pH 6.5, pH 7.4 and pH 10.0) equally. The drug release behavior was evaluated by the change of color and fluorescence of the release medium (Fig. 4C). In pH 5.0 PBS treated group, the color of the medium gradually deepened with the increase of time, and the microneedles were completely dissolved within 12 h. However, the pH 7.4 and pH 10.0 PBS treated group did not observe significant color change, and the microneedles maintained insoluble within 12 h. These results revealed that the acylhydrazone bond in the microneedles could be broken under acidic conditions, resulting in the dissolving of the microneedles to simultaneously release the loaded drugs. The fluorescence of the release medium was consistent with that of color change,
with the strongest fluorescence signal observed in pH 5.0 PBS treated group after treatment for 12 h.

In addition, a quantitative maps of the acid triggered drug release of microneedles was further investigated. As shown in Fig. 4D, at pH 7.4 PBS, ignorable release behavior was detected, and the release was as low as 7% during 18 h. A significant increase in release rate was observed at pH 6.5 PBS. In sharp contrast, when pH decreased to 5.0, the release rate was dramatically increased. The result suggested that the as-designed microneedles possessed pH-triggered drug release behavior. Since the microneedles was fabricated by crosslinking the aldehyde group of PA-Fe<sup>3+</sup> complex (Fe reservoir) and the hydrazide bond of HA-ADH-PpIX via forming acylhydrazone bond, upon triggered by acid pH, the pH-cleavable acylhydrazone bond can be broken, the hydrogel-based microneedles can transform from hydrogels to solution, thus HA-ADH-PpIX and PA-Fe complex can be released. Meanwhile, accompanied by the microneedles transform from hydrogels to solution, the loaded DHA may be rapidly released simultaneously. Therefore, for convenience of detection, the cumulative release rate of Fe was determined to represent the pH-triggered drug release behavior of microneedles.

The above results indicated that the pH-responsive microneedles have been successfully prepared, which were expected to be dissolved after inserted into the acidic tumor microenvironment and then release drug simultaneously for enhanced tumor therapeutic effect with minimized systemic toxicity.

### 3.5. Mechanical strength, skin insertion and penetration efficiency of microneedles

The pressure-displacement curve of the hydrazone bond cross-linked microneedle was exhibited in Fig. 5A. The microneedle could bear 76 N per array when its displacement was 400 μm, which far exceeded the force required for insertion into the skin [53, 54], suggesting that the as-designed microneedles have excellent mechanical strength. In addition, when the pressure on the microneedle gradually increased to 90 N, the tip of the microneedle was bent without break (Fig. 5B), indicating that the microneedle not only has good mechanical property, but also has excellent ductility, which was not easy to be destroyed.

Inspired by the excellent mechanical strength, the skin insertion capability of the microneedles was conducted.
After puncturing into the depilated isolated skin for 10 min and removing the microneedle, orderly and clear pinholes in the surface of skin were observed (Fig. 5C), suggesting the microneedle was successfully inserted into the skin. Comparing the number of effective puncture pinholes with the total number of microneedles (121) prepared, the puncture rate was calculated to be higher than 85%. The H&E staining image of skin cryo-section exhibited that the microneedles could insert into a depth of approximately 317 μm (Fig. 5D). Since cutaneous melanoma was caused by the abnormal rapid proliferation of melanocytes cross epidermis and dermis [55]. Reports summarized that the thickness of the epidermis is about 100–150 μm and the thickness of the dermis is about 3–5 mm [56], so DHA@HP-Fe-MN penetrating about 300 μm can reach the location where the melanoma exists. However, for deep tumor located in subcutaneous or deeper tissue, current microneedles are difficult to reach, while the drugs released from the microneedles may penetrate into the deep tumor tissues.

Additionally, the C6-labeled fluorescent microneedles were pierced into the isolated skin and observed by CLSM using 3D reconstruction techniques. As shown in Fig. 5E, green fluorescence represent the tip of the microneedles, the array of fluorescence was quadrangular pyramid in shape, which was consistent with the microneedle sequence, and the puncture rate was calculated to be about 80%. Further, the drug penetration of microneedles in the tissues was investigated using C6-labeled fluorescent microneedles. As displayed in Fig. S3, compared with the position that without microneedles insertion, obvious pore channels were observed on the rat skin in the position where microneedles inserted, and the bright green fluorescence from C6 distributed surrounding the inserting sites and penetrate vertically in the skin away from the insertion sites, indicating that the drug loaded in the microneedles can be released and penetrate into the deep tissues.

The result of the in vivo skin insertion and recovery experiments were shown in Fig. 5F. After the mice treated with microneedles for 10 min, several micro-channels produced on the back of mice could be recovered within 20 min without redness or inflammation. The above results indicated that the microneedles could overcome the barrier of stratum corneum and deliver drugs with good biocompatibility.

To develop a successful transdermal drug delivery system for biomedical applications, microneedles should not only need to have certain mechanical strength to overcome the skin barrier, but also effectively release therapeutic agents to produce local or systemic therapeutic effects. At the meanwhile, the materials applied for microneedles preparation should have good biosafety and biodegradability.

To address the above issues, in this work, a hydrogel-based microneedles was fabricated by crosslinking biocompatible HA-ADH conjugate with aldehyde containing “PA-Fe3+ complex”. Meanwhile, PpIX was conjugated with HA-ADH to avert unexpected aggregation for efficient PDT. The designed microneedles formed by cross-linking hydrophilic polymer and small molecule cross-linker exhibited excellent mechanical strength, which can efficiently inserted into the skin. The acylhydrazone bond formed in the hydrogel can be cleaved in the acid tumor microenvironment or acid skin tissues, then the hydrogels-based microneedles can transform from hydrogels to solution, accompanied by the release of drug-containing microneedle matrix material and
the loaded therapeutic agents, finally concentrating the drugs at the tumor sites to avoid blood circulation and reduce toxicity to normal tissues. This concept of microneedles design provided new ideas for the construction of localized stimuli-responsive drug release systems.

3.6. Cellular uptake and cytotoxicity of DHA@HPFe-MN microneedles

After evaluated the physicochemical properties of microneedles, whether the drug released from microneedles could be uptaken by tumor cells and effectively kill them were further investigated. Fe^{3+} red fluorescence probe was used to evaluate the cellular uptake of microneedles. According to the CLSM results (Fig. 6A), with the incubation time increased from 4 to 8 h, the red fluorescence in B16 cells gradually increased, indicating that PA-Fe^{3+} could be uptaken into cells and release Fe^{3+} under pH 6.5 culture medium, thus providing intercellular iron source.

Next, the cytotoxicity of free drug and free drug combination against B16 cells was investigated. DHA, a derivation of artemisinin, has an internal peroxy bridge structure, which can be broken under the catalysis of metal ions, such as Fe^{2+}, Mn^{2+} and Ni^{2+}, leading to ROS generation and cell death [22]. As shown in Fig. 6B, DHA showed a limited cytotoxic effect to B16 cells. When the concentration of DHA increased from 3.125 μM to 200 μM, the cell viability of B16 cells was about 58% and no longer increased. This
might be because the cytotoxicity of DHA was metal ions-dependent, while the Fe ions in tumor cells were limited. Then, the Fe-augmented chemotherapy effect of DHA was evaluated by incubating PA-Fe$^{3+}$ with DHA. PA-Fe$^{3+}$ showed little cytotoxic effect, the cell viability was greater than 80% when PA-Fe$^{3+}$ up to 500 μM after 24 h incubation (Fig. 6C). However, significantly enhanced cytotoxic effect was observed when co-incubated DHA with PA-Fe$^{3+}$. After 24-h incubation, the cell viability rate of 200 μM DHA plus 250 μM PA-Fe$^{3+}$ or 500 μM PA-Fe$^{3+}$ were 38% and 17% respectively, which were obviously lower than single DHA or PA-Fe$^{3+}$, respectively (Fig. 6D). The enhanced cytotoxicity of DHA plus PA-Fe$^{3+}$ might be contributed to that PA-Fe$^{3+}$ could be reduced by intracellular GSH to generate Fe$^{2+}$, which served as exogenous iron reservoir to activate the cytotoxic effect of DHA. Further, we evaluated the cytotoxicity of microneedles against B16 cells with or without irradiation under normoxia or hypoxia conditions. As shown in Fig. 6E, the light and HPFe-MN treated groups exhibited negligible cell-killing effect under normoxia conditions, indicating light irradiation and microneedle matrix material had good biocompatibility. Compared with HPFe-MN and DHA@MN groups, DHA@HPFe-MN showed stronger cell killing effect, indicating that Fe iron can effectively enhance the chemotherapy effect of DHA.
However, the cell viability of HPFe-MN + light was significantly decreased, indicating the highly effective PDT effect of the microneedle. In sharp contrast, DHA@HPFe-MN + light exhibited the strongest cytotoxic effect, possibly due to the combination of PDT and Fe-augmented DHA-mediated chemotherapy. By contrast, cells under hypoxia conditions and without light treatment showed similar results to that of normoxia conditions, indicating that DHA-based oxidative therapy was independent of oxygen. However, cells under hypoxia and with light treatment showed significantly compromised cell-killing effects compared with normoxia, suggesting the PDT effect could be attenuated when lack of oxygen (Fig. 6F). The Live/dead staining and apoptosis results under normoxia conditions were consistent with MTT. DHA@HPFe-MN + light displayed the largest amount of dead cells (red fluorescence) and almost no live cells (green fluorescence), indicating the strongest antitumor effect (Fig. 6G). Additionally, DHA@HPFe-MN + light induced the highest apoptotic cells (78.7%), which was higher than single chemotherapy (46.5%), Fe-augmented chemotherapy (56.7%) and single PDT (63.8%) (Fig. 6H and S4). All of these results suggested that compared with single chemotherapy or PDT, the combination of PDT and Fe-activated chemotherapy mediated by DHA@HPFe-MN could remarkably enhance the antitumor effect.

3.7. Intracellular ROS generation of microneedles

To elucidate the possible mechanism of DHA@HPFe-MN-mediated combined chemo-photodynamic therapy, intracellular ROS generation of microneedles was detected using DCFH-DA as ROS indicator. As shown in Fig. 7A, compared with control group, DHA@MN exhibited obvious fluorescence signal, indicating that DHA could generate ROS intracellular. By contrast, the fluorescence signal of DHA@HP-Fe-MN was significantly enhanced, suggesting that Fe could augment DHA-mediated oxidation therapy. HP-Fe-MN + light (PDT alone) exhibited a moderate green
Fig. 8 – (A) Tumor volume curve of B16 tumor-bearing mice after different treatments. (B) Photograph of the excised tumors and (C) Average tumor weights after different treatments. (D) H&E staining (Scale bar = 200 μm) and (E) TUNEL staining (Scale bar = 50 μm) of the excised tumors after various treatment. (F) Body weight changes of tumor-bearing mice after different treatments. (G) H&E staining of major organs of tumor-bearing mice after different treatments (Scale bar = 200 μm). Data were presented as mean ± SD (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001.
fluorescence, indicating that PpIX could produce abundant ROS during the PDT process. In sharp contrast, DHA@HPFe-MN + light displayed the strongest green fluorescence, indicating that the combined ROS generation effect of chemo-photodynamic therapy mediated by Fe-activated DHA and PDT in normoxia condition.

Since hypoxia is one of the typical characteristics of tumor microenvironment, and inadequate oxygen supply can significantly limit the efficiency of oxygen dependent PDT [57]. Then, the ability of microneedles to produce ROS in tumor hypoxic microenvironment was investigated. We used COCl₂ to induce the hypoxia model of B16 cells and treated them with different microneedles formulations. As shown in Figs. 7A-7B, similar green fluorescence signal appeared in DHA@MN and DHA@HPFe-MN treated groups no matter in normoxic and hypoxic B16 cells, indicating that the Fe-augmented DHA based oxidation treatment could produce ROS no matter under normoxic or hypoxic conditions. However, compared with the cells treated under normoxia, the ROS generation capacity of HP-Fe-MN + light (PDT alone) group and DHA@HPFe-MN + light group were significantly decreased under hypoxic conditions, indicating that hypoxia could greatly weaken the efficacy of PDT.

The above results indicated that DHA@HPFe-MN-mediated chemo-photodynamic therapy could remarkably amplify intracellular oxidative stress levels to induce cell death. In addition, though the PDT effect could be partly compromised in hypoxia tumor microenvironment, the Fe-activated DHA-based chemotherapy possessed the great potential to overcome the limitations of oxygen-dependent PDT-mediated therapy, enabling DHA@HPFe-MN with superior therapeutic effect.

3.8. In vivo antitumor efficiency

To evaluate the antitumor effect of DHA@HPFe-MN in vivo, a B16 tumor-bearing mouse model was developed. When the tumor volume reached about 100–150 mm³, the tumor-bearing mice were randomly divided into 7 groups and treated with different microneedles formulations. As shown in Fig. 8A, the tumor volume in saline group was the largest compared with other groups, and there was no significant difference between the saline and the saline + light group, indicating that light irradiation had no inhibitory effect on tumor growth. HPFe-MN exhibited little antitumor effect compared with saline group. DHA@MN showed a slightly enhanced antitumor effect compared with HPFe-MN, indicating that DHA could inhibit tumor growth to a little bit. By contrast, the tumor volume of DHA@HPFe-MN group was significantly inhibited, indicating that Fe could significantly enhance the antitumor effect of DHA. The remarkably enhanced antitumor effect compared with the saline group might be caused by that DHA and PA-Fe³⁺ loaded in DHA@HPFe-MN could be released in acidic tumor microenvironment, then uptaken by tumor cell to effectively inhibit tumor cells. HPFe-MN + light group showed moderate tumor inhibitory effect, indicating that PDT could effectively inhibit melanoma. Excitingly, the tumor growth of DHA@HPFe-MN + light group was almost completely inhibited, with tumor volume reduced to half of the initial treatment. The result indicated that the combination of PDT and Fe-activated chemotherapy effect of DHA could dramatically inhibit tumor growth, which was stronger than single chemotherapy and PDT alone. The significantly enhanced antitumor effect of DHA@HPFe-MN + light group might be caused by the amplification of intracellular oxidative stress in normoxia and hypoxia tumor microenvironments. The photograph and tumor weight of the excised tumors exhibited the similar trend to that of tumor volume (Fig. 8B-8C), which further verified the excellent antitumor effect of chemo-photodynamic therapy of DHA@HPFe-MN + light group. H&E and TUNEL staining further confirmed that DHA@HPFe-MN + light group induced the most remarkable apoptosis and necrosis of tumor cells (Fig. 8D-8E). All of these results suggested the remarkable chemo-photodynamic therapy induced by DHA@HPFe-MN + light that effectively eliminate the tumor cells.

Additionally, no evident body weight loss of all treatment groups was monitored (Fig. 8F), revealing negligible systemic toxicities of the combined Fe-augmented chemo-photodynamic therapy. Simultaneously, at the terminal of the antitumor experiment, major organs including hearts, livers, spleens lungs and kidneys were harvested. H&E staining of major organs observed that no obvious pathological abnormalities or damage in DHA@Fe-MN and DHA@HPFe-MN + light group when compared with the saline group, suggesting the excellent biocompatibility of the microneedles formulations (Fig. 8G). These results verified that DHA@HPFe-MN with excellent biosafety could be served as a potential microneedle-based localized drug reservoir for remarkable combined chemo-photodynamic therapy of epidermal tumors with minimized systemic toxicity.

4. Conclusion

In summary, we engineered a pH-responsive DHA@HPFe-MN microneedles as a localized drug delivery vehicle, which achieved pH-triggered drug release and localized synergistic chemo-photodynamic therapy of melanoma via oxidative stress amplification both in normoxia and hypoxia conditions. DHA@HPFe-MN was fabricated by crosslinking the photosensitizer-carrying microneedle matrix material HA-ADH-PpIX with “iron reservoir” PA-Fe³⁺ complex in the needle tip via the formation of pH-responsive acylhydrazone bond, and DHA was simultaneously loaded in the hydrogel network. HA-ADH-PpIX that conjugated PpIX to HA prevent the undesired aggregation of PpIX to guarantee the enhanced PDT effect, as evidenced by the generating of abundant ROS in vitro and intracellular. The as-designed DHA@HPFe-MN microneedles with quadrangular pyramid-like shape, sharp needle tip, efficient drug loading, and excellent mechanical stability could efficiently overcome skin barrier to deliver drug into the tumor site, where the acidic tumor environment triggered the degradation of microneedles to enable the Fe-activated DHA based chemotherapy. Under light irradiation, a combined chemo-photodynamic therapeutic effect was achieved via oxidative stress amplification. Noteworthy, the ROS generated by DHA under Fe catalyst was oxygen-independent, overcoming the oxygen-dependent effect of PDT to entirely eliminate tumor cells in normoxia and hypoxia.
tumor microenvironment. The versatile pH-activatable microneedles with excellent biosafety and biodegradability, is expected to be an attractive topical drug reservoir for combined chemo-photodynamic therapy of melanoma with minimized systemic toxicity.

Conflicts of interest

The author declares that there is no commercial interest or related interest that conflicts with the submitted work.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ajps.2022.08.003.

REFERENCE

[1] Kawczyk-Krupka A, Bugaj AM, Latos W, Zaremba K, Sieróń A. Photodynamic therapy in treatment of cutaneous and choroidal melanoma. Photodermag Photodyn 2013;10(4):503–9.
[2] Cassano R, Caonato M, Calviello G, Serini S, Trombino S. Recent advances in nanotechnology for the treatment of melanoma. Molecules 2021;26(4):785.
[3] Champeau M. Dissolving microneedles for an optimal transdermal delivery of an active principle used in photodynamic therapy: development and proof of concept. Université du Droit et de la Santé-Lille II; 2020.
[4] Huang S, Liu H, Huang S, Fu T, Xue W, Guo R. Dextran methylacrylate hydrogel microneedles loaded with doxorubicin and trametinib for continuous transdermal administration of melanoma. Carbohydr Polym 2020;246:116650.
[5] Domingues B, Lopes JM, Soares P, Pópolo H. Melanoma treatment in review. Immunotargets Ther 2018;7:35–49.
[6] Song G, Sun Y, Liu T, Zhang X, Zeng Z, Wang R, et al. Transdermal delivery of Cu-doped polydopamine using microneedles for photothermal and chemodynamic synergistic therapy against skin melanoma. Chem Eng J 2021;426:130790.
[7] Bennassar A, Ishioka P, Vilalta A. Surgical treatment of primary melanoma. Dermatol Ther 2012;25(5):432–42.
[8] Zou Z, Chang H, Li H, Wang S. Induction of reactive oxygen species: an emerging approach for cancer therapy. Apoptosis 2017;22(11):1521–35.
[9] Liu C, Cao Y, Cheng Y, Wang D, Xu T, Su L, et al. An open source and reduce expenditure ROS generation strategy for chemodynamic/photodynamic synergistic therapy. Nat Commun 2020;11(1):1–9.
[10] Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov 2009;8(7):579–91.
[11] Bian Q, Huang L, Xu Y, Wang R, Gu Y, Yuan A, et al. A facile low-dose photosensitizer-incorporated dissolving microneedles-based composite system for eliciting antitumor immunity and the abscopal effect. ACS Nano 2021;15(12):19468–79.
[12] Deepagan V, You DG, Um W, Ko H, Kwon S, Choi KY, et al. Long-circulating Au-TiO2 nanocomposite as a sonosensitizer for ROS-mediated eradication of cancer. Nano Lett. 2016;16(10):6257–64.
[13] Zhang M, Qin X, Xu W, Wang Y, Song Y, Garg S, et al. Engineering of a dual-modal phototherapeutic nanoplatform for single NIR laser-triggered tumor therapy. J Colloid Interf Sci 2021;594:493–501.
[14] Xi D, Xu N, Xia X, Shi C, Li X, Wang D, et al. Strong π-π stacking stabilized nanophotosensitizers: improving tumor retention for enhanced therapy for large tumors in mice. Adv Mater 2022;34(6):2106797.
[15] Huang Y, Xiao Z, Guan Z, Shen Y, Jiang Y, Xu X, et al. A light-triggered self-reinforced nanogent for targeted chemo-photodynamic therapy of breast cancer bone metastases via ER stress and mitochondria mediated apoptotic pathways. J Control Release 2020;319:119–34.
[16] Huang Y, Guan Z, Dai X, Shen Y, Wei Q, Ren L, et al. Engineered macrophages as near-infrared light activated drug vectors for chemo-photodynamic therapy of primary and bone metastatic breast cancer. Nat Commun 2021;12(1):1–22.
[17] Ng SY, Kamkaw A, Fu N, Kue CS, Chung LY, Kiew LV, et al. Active targeted ligand-aza-BODIPY conjugate for near-infrared photodynamic therapy in melanoma. Int J Pharmaceut 2020;579:119189.
[18] Zhou Z, Zhang L, Zhang Z, Liu Z. Advances in photosensitizer-related design for photodynamic therapy. Asian J Pharm Sci 2021;16(6):668–86.
[19] Bai G, Gao Y, Liu S, Shui S, Liu G. pH-dependent rearrangement determines the iron-activation and antitumor activity of artemisinins. Free Radical Bio Med 2021;163:234–42.
[20] Bai S, Lu Z, Jiang Y, Shi X, Xu D, Shi Y, et al. Nanotransferrin-based programmable catalysis mediates three-pronged induction of oxidative stress to enhance cancer immunotherapy. ACS Nano 2021;16(1):997–1012.
[21] Dong L, Wang C, Zhen W, Jia X, An S, Xu Z, et al. Biodegradable iron-coordinated hollow polydopamine nanospheres for dihydroartemisinin delivery and selectively enhanced therapy in tumor cells. J Mater Chem B 2019;7(40):6172–80.
[22] Liu L, Wei Y, Zhai S, Chen Q, Xing D. Dihydroartemisinin and transferrin-dual-dressed nano-graphene oxide for a pH-triggered chemotherapy. Biomaterials 2015;62:35–46.
[23] Zhang X, Yang S, Wang Q, Ye W, Liu S, Wang, et al. Tailored theranostic nanoparticles cause efficient ferroptosis in head and neck squamous cell carcinoma through a reactive oxygen species "butterfly effect. Chem Eng J 2021;423:130083.
[24] Yu XA, Lu M, Luo Y, Hu Y, Zhang Y, Xu Z, et al. A cancer-specific activatable theranostic nanodrug for enhanced therapeutic efficacy via amplification of oxidative stress. Theranostics 2020;10(1):371.
[25] Xiao Y, Zhang T, Ma X, Yang QC, Yang LL, Yang SC, et al. Microenvironment-responsive prodrug-induced pyroptosis boosts cancer immunotherapy. Adv Sci 2021;8(24):2101840.
[26] Allen TM, Cullis PR. Drug delivery systems: entering the mainstream. Science 2004;303(5655):1818–22.
[27] Mu W, Chu Q, Liu Y, Zhang N. A review on nano-based drug delivery system for cancer chemoimmunotherapy. Nano-Micro Lett 2020;12(1):1–24.
[28] Gilani SJ, Jahangir MA, Rizwanullah M, Taleuzzaman M, Shahab MS, Shakeel K, et al. Nano-based therapy for treatment of skin cancer. Recent Pat Anti Infect Drug Discov 2018;13(2):151–63.

[29] Saravanan M, Poornima S, Karthik V, Vigneshwaran A, Manikandan S, Ramasamy S, et al. Emerging nano-based drug delivery approach for cancer therapeutics. Handbook of research on nano-strategies for combating antimicrobial resistance and cancer. 2021; p. 271–93.

[30] Li Q, Zhou Y, He W, Ren X, Zhang M, Jiang Y, et al. Platelet-armored nanoplatform to harmonize janus-faced IFN-γ against tumor recurrence and metastasis. J Control Release 2021;358:33–45.

[31] Ma X, Li SJ, Liu Y, Zhang T, Xue P, Kang Y, et al. Bioengineered nanogels for cancer immunotherapy. Chem Soc Rev 2022;51:5136–74.

[32] Xu S, Liu C, Zang S, Li J, Wang Y, Ren K, et al. Multifunctional self-delivery micelles targeting the invasion-metastasis cascade for enhanced chemotherapy against melanoma and the lung metastasis. Asian J Pharm Sci 2021;16(6):794–805.

[33] Kemp JA, Shim MS, Heo CY, Kwon YJ. Combo* nanomedicine: co-delivery of multi-modal therapeutics for efficient, targeted, and safe cancer therapy. Adv Drug Deliver Rev 2016;98:3–18.

[34] He M, Wang R, Wan P, Wang H, Cheng Y, Miao, et al. Biodegradable Ru-containing polycarbonate micelles for photoinstructed anticancer multitherapeutic agent delivery and phototherapy enhancement. Biomacromolecules 2022;23(4):1733–44.

[35] Sun W, Li S, Häupler B, Liu J, Jin S, Steffen W, et al. An amphiphilic ruthenium polynometallogrid for combined photodynamic therapy and photochemistry in vivo. Adv Mater 2017;29(6):1603702.

[36] He M, He G, Wang P, Jiang S, Jiao Z, Xi D, et al. A sequential dual-model strategy based on photoactivatable metallopolymers for on-demand release of photosensitizers and anticancer Ddrugs. Adv Sci 2021;8(23):2103334.

[37] Li G, Sun B, Zheng S, Xu L, Tao W, Zhao D, et al. Zwitterion-driven shape program of prodrug nanoassemblies with high stability, high tumor accumulation, and high antitumor activity. Adv Healthc Mater 2021;10(23):2170115.

[38] Shi J, Kantoff PW, Wooster R, Farokhzad OC. Cancer nanomedicine: progress, challenges and opportunities. Nat Rev Cancer 2017;17(1):20–37.

[39] Wilhelm S, Tavares AJ, Dai Q, Ohta S, Audet J, Dvorak HF, et al. Analysis of nanoparticle delivery to tumours. Nat Rev Mater 2016;1(5):1–12.

[40] Chen SX, Ma M, Xue F, Shen S, Chen Q, Kuang Y, et al. Construction of microneedle-assisted co-delivery platform and its combining photodynamic/immunotherapy. J Control Release 2020;324:218–27.

[41] Alimardani V, Abolmaali SS, Tamaddon AM, Ashfaq M. Recent advances on microneedle arrays-mediated technology in cancer diagnosis and therapy. Drug Deliv Transl Re 2020;11:788–816.

[42] Jamaledin R, Makvandi P, Yiu CK, Agarwal T, Vecchione R, Sun W, et al. Engineered microneedle patches for controlled release of active compounds: recent advances in release profile tuning. Advanced Therapeutics 2020;3(12):2000171.

[43] Yang L, Yang Y, Chen H, Mei L, Zeng X. Polymeric microneedle-mediated sustained release systems: design strategies and promising applications for drug delivery. Asian J Pharm Sci 2022;17(1):70–86.

[44] Li D, Hu D, Xu H, Patra HK, Liu X, Zhou Z, et al. Progress and perspective of microneedle system for anti-cancer drug delivery. Biomaterials 2021;264:120410.

[45] Hao Y, Chen Y, He X, Yang F, Han R, Yang C, et al. Near-infrared responsive 5-fluorouracil and indocyanine green loaded MPEG-PCL nanoparticle integrated with dissolvable microneedle for skin cancer therapy. Bioact Mater 2020;5(3):542–52.

[46] Zhou Z, Zhang S, Yang G, Cao Y. Enhanced delivery efficiency and sustained release of biopolymers by complexation-based gel encapsulated coated microneedles: rhIFN-1b example. Asian J Pharm Sci 2021;16(5):612–22.

[47] Xu X, Zeng Z Huang, Sun Z, Huang Y, Chen Y, Ye J, et al. Near-infrared light-triggered degradable hyaluronic acid hydrogel for on-demand drug release and combined chemo-photodynamic therapy. Carbohydr Polym 2020;229:115394.

[48] Yu F, Cao X, Du J, Wang G, Chen X. Multifunctional hydrogel with good structure integrity, self-healing, and tissue-adhesive property formed by combining Diels–Alder click reaction and acylhydrazone bond. ACS Appl Mater Inter 2015;7(43):24023–31.

[49] Prestwich GD, Marecek DM, Marecek JF, Vercruyssse KP, Ziebell MR. Controlled chemical modification of hyaluronic acid: synthesis, applications, and biodegradation of hyaluronic acid derivatives. J Control Release 1998;53(1–3):93–103.

[50] Ceylan H, Urel M, Erkal TS, Tekinay AB, Dana A, Guler MO. Mussel inspired dynamic cross-linking of self-healing peptide nanofiber network. Adv Funct Mater 2013;23(16):2081–90.

[51] Huang Z, Wei G, Zeng Z, Huang Y, Huang L, Shen Y, et al. Enhanced cancer therapy through synergetic photodynamic/immune checkpoint blockade mediated by a liposomal conjugate comprised of porphyrin and IDO inhibitor. Theranostics 2019;9(19):5542.

[52] Hruby M, Koňák Č, Ulbrich K. Polymeric micellar pH-sensitive drug delivery system for doxorubicin. J Control Release 2005;103(1):137–48.

[53] Ma G, Wu C. Microneedle, bio-microneedle and bio-inspired microneedle: a review. J Control Release 2017;251:11–23.

[54] Gittard SD, Chen B, Xu H, Ovsianikov A, Chichkov BN, Monteiro-Riviere NA, et al. The effects of geometry on skin penetration and failure of polymer microneedles. J Adhes Sci Technol 2013;27(3):227–43.

[55] Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature 2007;445(7130):851–7.

[56] Yang D, Chen M, Sun Y, Jin Y, Lu C, Pan X, et al. Microneedle-mediated transdermal drug delivery for treating diverse skin diseases. Acta Biomater 2021;121:119–33.

[57] Zhang C, Qin WJ, Bai XF, Zhang XZ. Nanomaterials to relieve tumor hypoxia for enhanced photodynamic therapy. Nano Today 2020;35:100960.