Iron ion and siramesine-loaded polydopamine nanoparticles for enhanced breast cancer therapy

Yiming Wang

Abstract

Ferroptosis is a novel type of nonapoptotic cell death, showing a hopeful potential in cancer therapeutic schedules. One of the apparent features of ferroptosis is the lipid hydroperoxides producing ROS induced by intracellular iron ions accumulation. Thus the iron regulation and metabolism in the cell is an essential factors for the trigger of ferroptosis. We designed a new nanoparticle that loaded ferric ions and siamese based on polydopamine nanomaterial (siramesine@ Fe(III) PDA NPs) to induce breast cancer ferroptosis through the Fenton reaction. Iron ions can be added to cancer cells, and siamese can block the exportation of intracellular iron by inhibiting the expression of FPN, thus promoting the Fenton reaction. The extra transport of iron ions into cells by nanoparticles and the inhibition of iron ions outward by Siramesine promoted the accumulation of iron ions in cells, thus achieving a synergistic effect leading to ferroptosis in cells. In addition, NPs demonstrated excellent pH-responsive degradation, with enhanced drug release at the pH value of tumor microenvironments and lysosomal. Siramesine@Fe(III) PDA NPs showed good ferroptosis induction ability, displaying the potential for cancer treatment.

Keywords: Ferroptosis, Siramesine, Polydopamine, Ferroportin, Breast cancer therapy

1. Introduction

Breast cancer (BC) is the most common carcinoma among females and the fourth and second primary cause of mortality in carcinoma cases of all types of cancer and women's cancer around the world, respectively, with 1.7 million people diagnosed around the world and about 500 thousand people dying from this disease yearly [1,2]. Up to now, many therapies have been applied to treat breast cancer, including traditional surgical resection, radiotherapy, pharmacotherapy, hormonal therapy, and emerging targeted proapoptotic therapy [1,3,4]. The conventional use of pharmacotherapy remains a critical component in the treatment of breast cancer; however, long-term drug use-induced resistance to chemotherapy and the gradually intolerable side effect are critical issues in limiting chemotherapeutic agents [5,6chest wall, and regional lymph nodes. In some cases, these local treatments may prevent the dissemination of cancer and may reduce mortality from breast cancer. Cytotoxic chemotherapy and hormonal therapy are systemic treatments given after local treatment to reduce systemic recurrences and overall mortality from breast cancer. Recent guidelines from the National Institutes of Health Consensus Conference, the National Comprehensive Cancer Center Network, and other groups recommend adjuvant chemotherapy, tamoxifen, . . .",”container-title”:”New England Journal of Medicine”,”DOI”:”10.1056/NEJM200106283442607”,”ISSN”:”0028-4793”,”issue”:”26”,”note”:”publisher: Massachusetts Medical Society\n_eprint: https://doi.org/10.1056/NEJM200106283442607\nPMID: 11430330”,”page”:”1997-2008”,”source”:”Taylor and Francis+NEJM”,”title”:”Side Effects of Adjuvant Treatment of Breast Cancer”,”volume”:”344”,”author”:[{“family”:”Shapiro”,”given”:”Charles L.”},{“family”:”Recht”,”given”:”Abram”}],”issued”:{“date-parts”:[[“2001”,6,28]]}},{“id”:353,”uris”:[“http://zotero.org/users/10526562/items/I8RCDM6N”],”itemData”:{“id”:353,”type”:”article-journal”,”abstract”:”Recent developments with chemotherapy for breast cancer have improved patient survival. However, there continue to be nonresponders to conventional anticancer agents. Multidrug resistance (MDR]. Although endocrine therapy targeting the impacts of estrogen has been clinically proven to reduce breast cancer mortality and recurrence rate, its efficacy is limited by intrinsic and acquired therapeutic resistance [7]. The reconstruction of breast cancer metabolism worsened the tumor microenvironment (TME) response to therapy-related cell death, contributing to mortality in breast cancer patients [8]. Despite extensive research on targeting apoptotic death of cancer cells, the clinical implementation of relevant therapeutic drugs is still a long way off [9]. Indeed, there is evidence to demonstrate that cancer cells have the ability to be resistant to apoptotic cellular death [10]. Therefore, there is an urgent need to develop emerging therapy with a nonapoptotic pathway for breast cancer patients.

Initially suggested by Stockwell and colleagues in 2012, ferroptosis is an emerging form of nonapoptotic cell death marked by an iron-dependent gathering of lipid hydroperoxides, leading to the production of lethal levels of reactive oxygen species (ROS) [11,12]. The significant mechanisms of ferroptosis are invariably associated with the obstacle of ferric metabolism-induced iron accumulation and consequent lipid peroxidation [13]. In the second place, cystine/glutamate antiporter systems Xc^- and glutathione peroxidase 4 (GPX4) are two essential regulatory factors referring to lipid peroxidation and cellular antioxidant capacity [14]. Inhibition of the activity of the Xc system leads to down-regulating the synthesis of GSH by inhibiting the uptake of cysteine, resulting in depletion of the essential intracellular antioxidant GSH, which serves as a reducing co-substrate for GPX4, shielding cells from severe oxidative damage created by the highly toxic ROS. GPX4 activity can be rendered inactive, leading to the buildup of ROS in cancer cells, as a result of GSH depletion [14,15but not of 11 compounds with other lethal mechanisms. In addition, two representative ferroptosis inducers prevented tumor growth in xenograft mouse tumor models. Sensitivity profiling in 177 cancer cell lines revealed that diffuse large B cell lymphomas and renal cell carcinomas are particularly susceptible to GPX4-regulated ferroptosis. Thus, GPX4 is an essential regulator of ferroptotic cancer cell death.","container-title":"Cell","DOI":"10.1016/j.cell.2013.12.010","ISSN":"0092-8674","issue":"1","journalAbbreviation":"Cell","language":"en","page":"317-331","source":"ScienceDirect","title":"Regulation of Ferroptotic Cancer Cell Death by GPX4","volume":"156","author":[{"family":"Yang","given":"Wan Seok"},{"family":"SriRamaratnam","given":"Rohitha"},{"family":"Welsch","given":"Matthew E."},{"family":"Shimada","given":"Kenichi"},{"family":"Skouta","given":"Rachid"},{"family":"Viswanathan","given":"Vasanthi S."},{"family":"Cheah","given":"Jaime H."},{"family":"Clemons","given":"Paul A."},{"family":"Shamji","given":"Alykhan F."},{"family":"Clish","given":"Clary B."},{"family":"Brown","given":"Lewis M."},{"family":"Girotti","given":"Albert W."},{"family":"Cornish","given":"Virginia W."},{"family":"Schreiber","given":"Stuart L."},{"family":"Stockwell","given":"Brent R."}],"issued":{"date-parts":[["2014",1,16]]}}},{"id":365,"uris":["http://zotero.org/users/10526562/items/N4XCCLH2"],"itemData":{"id":365,"type":"article-journal","abstract":"The past decade has yielded tremendous insights into how cells die. This has come with our understanding that several distinct forms of cell death are encompassed under the umbrella term necrosis. Among these distinct forms of regulated necrotic cell death, ferroptosis has attracted considerable attention owing to its putative involvement in diverse pathophysiological processes. A key feature of the ferroptosis process is the requirement of phospholipid peroxidation, a process that has been linked with several human pathologies. Now with the establishment of a connection between lipid peroxidation and a distinctive cell death pathway, the search for new small molecules able to suppress lipid peroxidation has gained momentum and may yield novel cytoprotective strategies. We review here advances in our understanding of the ferroptotic process and summarize the development of lipid peroxidation inhibitors with the ultimate goal of suppressing ferroptosis-relevant cell death and related pathologies.","container-title":"Trends in Pharmacological Sciences","DOI":"10.1016/j.tips.2017.02.005","ISSN":"0165-6147","issue":"5","journalAbbreviation":"Trends in Pharmacological Sciences","language":"en","page":"489-498","source":"ScienceDirect","title":"Ferroptosis Inhibition: Mechanisms and Opportunities","title-short":"Ferroptosis Inhibition","volume":"38","author":[{"family":"Angeli","given":"Jose Pedro Friedmann"},{"family":"Shah","given":"Ron"},{"family":"Pratt","given":"Derek A."},{"family":"Conrad","given":"Marcus"}],"issued":{"date-parts":[["2017",5,1]]}}}],"schema":"https://github.com/citation-style-language/schema/raw/master/csl-citation.json"}]. Furthermore, Xc system inhibiter can inhibit the synthesis of new GSH by inactivating the cystine/cysteine redox cycle and restricting the supply of cysteine in the cell, and lead to cancer cell ferroptosis death [16]. Meanwhile, GPX4 is also a crucial focus in the context of ferroptosis because its incapacitation prevents the breakdown of lipid peroxides, thereby avoiding the accumulation of ROS and the extensive consequential damage of various cellular molecules such as nucleic acids, proteins, and lipids [17]. The excess iron within cells can trigger ferroptotic cell death by catalyzing intracellular H_2O_2 through the Fenton reaction, which produces highly poisonous hydroxyl radicals (OH) and leads to the oxidation of lipids, proteins, and DNA [18,19the dual nature of both iron and reactive oxygen species (ROS]. The morphology of ferroptosis is quite different from the other traditional shapes of cellular program death, such as apoptosis, necrosis, and autophagy: its hallmark features comprise a decrease in cellular volume, an increase in mitochondrial membrane density, the disappearance or reduction of mitochondrial cristae, and the rupture of the outer mitochondrial membrane [13,20particularly lipid hydroperoxides. This form of iron-dependent cell death is genetically, biochemically, and morphologically distinct from other cell death modalities, including apoptosis, unregulated necrosis, and necroptosis. Ferroptosis

is regulated by specific pathways and is involved in diverse biological contexts. Here we summarize the discovery of ferroptosis, the mechanism of ferroptosis regulation, and its increasingly appreciated relevance to both normal and pathological physiology.",”container-title”:”Trends in Cell Biology”,”DOI”:”10.1016/j.tcb.2015.10.014”,”ISSN”:”0962-8924”,”issue”:”3”,”journalAbbreviation”:”Trends in Cell Biology”,”language”:”en”,”page”:”165-176”,”source”:”ScienceDirect”,”title”:”Ferroptosis: Death by Lipid Peroxidation”,”title-short”:”Ferroptosis”,”volume”:”26”,”author”:[{“family”:”Yang”,”given”:”Wan Seok”},{“family”:”Stockwell”,”given”:”Brent R.”}],”issued”:{“date-parts”:[[“2016”,3,1]]}}},{“id”:316,”uris”:[“http://zotero.org/users/10526562/items/65QDXA4P”],”itemData”:{“id”:316,”type”:”article-journal”,”abstract”:”Ferroptosis is a recently recognized form of regulated cell death. It is characterized morphologically by the presence of smaller than normal mitochondria with condensed mitochondrial membrane densities, reduction or vanishing of mitochondria crista, and outer mitochondrial membrane rupture. It can be induced by experimental compounds (e.g., erastin, Ras-selective lethal small molecule 3, and buthionine sulfoximine]. To promote growth, cancer cells show a high dependency on iron compared to normal noncancerous cells, which makes them more susceptible to iron overloading-induced ferroptosis [21]. Therefore, ferroptosis has the development potent and hopeful in killing therapy-resistant cancers.

Iron also plays an essential role in the occurrence of Fenton reaction in vivo. In a variety of biological and pathological conditions, iron is considered an important initiator and mediator of iron-induced cell death [22,23]. Ferroportin (FPN) is the sole transporter responsible for eliminating iron ions from cells. It performs a vital role in the transmembrane export of iron and intracellular regulation of ferric homeostasis [24–26ferroportin 1 mutations leading to hemochromatosis (HFE4]. FPN plays a critical role in regulating iron acquisition, utilization and storage, so FPN-mediated iron export is highly monitored [23]. Changes in the expression level of FPN may lead to intracellular iron overload or deficiency [27–29SLC40A1, and MTP1]. Endocellular iron accumulation is associated with the dysfunction of iron export caused by low expression of FPN, which contributes to ferroptosis via increasing lipid ROS production by the Fenton reaction [30]. Interestingly, knockdown of FPN decreased the resistant ability to Erastin-induced ferroptosis of cells, whereas increased expression of FPN can enhance cell resistance to ferroptosis [31,32]. Meanwhile, evidence shows that after dealing with iron oxide nanoparticles (IO NPs), cells grow the expression of FPN in order to export iron to resist iron overload-induced ferroptosis [33]. Siramesine, a lysosome disrupting agent initially developed for depression, showed the ability to inhibit breast cancer by decreasing the expression of FPN to initiate ferroptosis [34,35].

Evidence has exhibited that cancer endocellular iron deficiency is vital in restraining the initiation of ferroptosis cell death; therefore, it is familiar to apply nanomaterial to deliver iron ions into the cancer cell [36]. The treatment of ferroptosis is not satisfactory due to the adverse catalytic occasion caused by tumor microenvironment (TME); therefore, some studies have applied other therapies in combination. IO NPs, for instance, have been coloaded with chemotherapeutics for synergistic chemotherapy therapy [37]. Polydopamine (PDA) is an ultrastable mussel-like biopolymer with biocompatibility structured with a rich benzene ring and catechol groups, which can load hydrophobic small molecule drugs via the π-π conjugation or hydrogen bonding, and metal ions through chelation interactions [38]. Based on this excellent physicochemical nature, PDA offers many functions related to cancer research and treatment, showing great potential in the biomedical field [39,40].

Herein, we report a ferroptosis therapeutic nanomaterial constituted by ferric iron and siramesine-loaded nanoparticles (siramesine@FeIIIPDA NPs) that have synergistic effects for tumor therapy. The siramesine@FeIIIPDA NPs, which induce ferroptosis in tumor cells, were obtained by loading ferric ions and siramesine on PDA through metal ion coordination interaction and π-π conjugation, respectively. Under the reaction of metal reductase in the intracellular, ferric ions were reduced to ferrous ions and then catalyzed intracellular H_2O_2 to produce highly toxic ·OH through the Fenton reaction. The released siramesine inhibits the expression of the sole iron export protein FPN to disable the cell's capacity to remove iron ions from the cell and lead to the accumulation of iron moved into the nanomaterial, eventually leading to a ferroptosis of a cancer cell.

2. Materials and methods

2.1 Reagents and materials

Sigma-Aldrich (Shanghai, China) was the source of Dopamine hydrochloride (DA·HCl), aqueous ammonia ($NH_3·H_2O$), and ferric chloride hexahydrate ($FeCl_3·6H_2O$). Siramesine was bought form MedChemExpress (Shanghai, China). Cell Counting Kit-8 (CCK-8) kit and Iron Assay Kits-Colorimetric were brought from Dojindo (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was brought from ATCC (Shanghai, China). Fetal bovine serum (FBS) were obtained from Atlanta Biologicals

(Shanghai, China). 4 ,6-Diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC) were purchased form Abcam (Shanghai, China). 2',7'-Dichlorodihydr ofluorescein diacetate (DCFH-DA), 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora3a,4a-diaza-s-indacene-3-undecanoic acid (C11BODIPY) and $Fe_4[Fe(CN)_6]_3$ (Prussian blue) were brought from Abcam (Shanghai, China). Calcein acetoxymethylester (Calcein-AM) and propidium iodide (PI) were obtained from Thermo Fisher Scientific (Shanghai, China).

2.2 Preparation of PDA NPs

DA·HCl 50 mg was added to the mixture of 9 mL deionized (DI) water and 4 mL ethanol alcohol. Then, the aqueous ammonia was slowly added to the mix to adjust the solution to mild alkaline and stirred for 24 h at 25 ℃. Through centrifuging at 15,000 g for 10 min, PDA NPs were gathered and washed three times with the mixture of anhydrous ethanol and water (v/v=1:1).

2.3 Preparation of siramesine@Fe(III) PDA NPs

Siramesine and 1 mg PDA NPs were added to 1mL ethanol in different w/w ratios. Then add 100 mg/mL, 0.1 mL $FeCl_3 \cdot 6H_2O$ solution to the mixture. In order to ensure the presence of Fe^{3+} ions, the liquid was maintained at a pH of 1.5 and then agitated at room temperature for 24h. After dialyzing (MWCO: 1 kDa) for 24 h, gather siramesine@Fe(III) PDA NPs. Other NPs (siramesine@PDA NPs, Fe(III)@PDA NPs) used in controlled experiments were prepared similarly.

2.4 Characterization of siramesine@Fe(III) PDA NPs

For measuring the loading degree (LD) and encapsulation efficiency (EE) of siramesine and ferric ions in the siramesine@Fe(III) PDA NPs. The content of siramesine in NPs was measured by High Performance Liquid Chromatography (HPLC, Agilent-1290). The mobile and stationary phases were determined on acetonitrile-0.1% formic acid (70:30) and C18 columns, respectively. The flow rate was set at 1mL/min and the detection wavelength was 250 nm. Then the Fe^{3+} ions content in NPs was measured by treating NPs with 1% HNO_3 solution with stirred for 24 h to make the iron sufficiently dissociate and measured by an inductively coupled plasma optical emission spectrometer (ICP-OES, Avio200). The LE and EE were determined as follows:

$$LE(\%) = \frac{siramesine\ weight\ or\ Fe^{3+}\ contents\ in\ NPs}{NPs\ weight} \times 100\ \%$$

$$EE(\%) = \frac{siramesine\ weight\ or\ Fe^{3+}\ contents\ in\ NPs}{total\ siramesine\ weight\ or\ FeCl_3} \times 100\%$$

Transmission electron microscopy (TEM, Tecnai G2 BioTWIN) and field emission scanning electron microscopy (FESEM, Nova NanoSEM) were used to examine the structure and dimensions of NPs. Dynamic light scattering (DLS, Malvern Zetasizer Nano S) was employed to measure the hydrated particle size distribution, polydispersity index (PDI), and zeta potential of various NPs.

2.5 In vitro release of siramesine and Fe

Siramesine release profile was determined by subjecting samples to dialysis at 37 ℃ in buffers with varying pH values (7.4, 6.5, and 5.0). Then the released content of siramesine was gathered and date by High-Performance Liquid Chromatography (HPLC, Agilent-1290) over the time change (1, 2, 4, 6, 12, 24, 36, 48 h).

so as to assess the release capacity of siramesine@Fe(III) PDA nanoparticles towards Fe ions, the nanoparticles were introduced into buffer solutions at various pH levels (pH 7.4, pH 6.5, and pH 5.0) and subjected to incubation at 37°C with shaking for a duration of 24 h. Treated by centrifuging at 15,000g for 10min, the sediment-removing solution with release Fe^{3+} ions were estimated by ICP-OES (Avio200).

2.6 Cell culture

MDA MB-231 breast tumor cell lines were cultured in a 5% CO2, 37°C incubator using Dulbecco's modified Eagle medium (DMEM) supplemented with 1% penicillin-streptomycin per ml and 10% fetal bovine serum (FBS).

2.7 Cellular uptake

Using FITC to label siramesine@Fe(III) PDA NPs for imaging, 1 mg FITC were mixed with 1mL NPs (2 mg/mL) added in DMSO and stirred for 12h. The siramesine@Fe(III) PDA NPs labelled with FITC were obtained after dialysis (MWCO: 3.5 kDa) for 24 h to dispart excess FITC, followed by washing with deionized water. To determine whether NPs were internalized into cells, the MDA-MB-231 cell was dealt with FITC-labeled siramesine@Fe(III) PDA NPs (0.2 mg/mL) for 1, 2, 4, and 6 h. Then cells were trypsinized to collect the suspensions and cells were dyed with DAPI for 10 min. Finally, the treated cells were observed by confocal laser scanning microscopy (CLSM, Olympus FV3000).

To research the behavior of cell uptake of siramesine@Fe(III) PDA NPs, the MDA-MB-231 cells were grown with FITC-labeled siramesine@Fe(III) PDA NPs at a concentration of 50 μg $mg^{-1}$for 1, 2, 4, and 6 h at 37 ℃.

Medium-removing cells were washed with PBS and resuspended in 0.5 mL PBS after trypsinizing for 1min, and a flow cytometer (FCM, BD FACSVerseTM) analyzed the cells.

2.8 Cell viability.

MDA-MB-231 cells were seeded onto 96-well plates (100 μL cell suspension per well) and cultured for 24 h. In order to verify the synergistic effect, the cells were incubated with different ingredients, including siramesine (40 μg/mL), $FeCl_3$ (iron ions: 40 μg/mL), siramesine@ PDA NPs (siramesine: 40 μg/mL), Fe(III)@PDA NPs (Fe^{3+} irons: 40 μg/mL) and siramesine@Fe(III) PDA NPs (siramesine: 40 μg/mL), and different NPs concentration (0, 5, 10, 20, 40, 80 μg/mL) after replacing new incubation media. To demonstrate the toxicity of siramesine@Fe(III) PDA NPs to breast tumour cells in a highly metabolic environment (excess ROS), we simulated TME with extra H_2O_2 to explore its cytotoxicity. The cells were washed with PBS and treated with 10μL CCK-8 solution at 37℃ for 1h. The absorbance of the samples was confirmed at 450nm wavelength by a microplate reader (Dynex MRX Revelation).

In the calcein-AM/PI staining experiment, MDA-MB-231 cells were cultured with various substances, including $FeCl_3$, siramesine, siramesine@ PDA NPs, Fe(III)@PDA NPs, and siramesine@Fe (III) PDA NPs at the content of 40 μg/mL for siramesine or Fe, in a 12-well microplate for 6 hours. Finally, Calcein-AM/PI staining was employed to visualize the cells, which were then imaged using CLSM.

2.9 Intracellular reactive oxygen species (ROS) detection

MDA-MB-231 cells were treated with different substances ($FeCl_3$, siramesine, Fe(III)@PDA NPs, siramesine@ PDA NPs, and siramesine@Fe (III) PDA NPs at the concentration of 40 μg mL^{-1} for siramesine or Fe) for 6h at 37℃. Subsequent to sediment removal, cells were cultured with DCFH-DA for 30 minutes to quantify ROS generation. After removing the excess probes with PBS, the resulting fluorescence emissions were visualized by CLSM and quantified by FCM.

2.10 The mechanism behind the combination.

To evaluate the intercellular concentration of iron ions, Prussian blue staining was utilized. After receiving different treatments, the cells were rinsed with PBS and then subjected to fixation with 4% paraformaldehyde solution for a duration of 30 minutes. After being washed 3 times with PBS, the cells were treated with Prussian blue solution (10mg/mL) for 30 minutes and then washed 3 times with PBS. The cells that were subjected to staining were observed under a light microscope to identify the intracellular localization of iron oxide.

For measuring intracellular iron, MDA-MB-231 cells were incubated with different groups at 37℃ for 6h. Cell lysates were collected by centrifugation for 10 min at 16,000g after washing with PBS. Then, the level of Fe in the supernatants was determined by the corresponding Iron Assay Kits-Colorimetric (Dojindo, Shanghai, China) according to the manufacturer's instructions.

Detecting the intracellular expression of FPN: MAD-MB-231 cells were seeded onto a 6-well plate and then incubated with different groups (siramesine, $FeCl_3$, siramesine@Fe(III) PDA NPs) for 24h. Cell lysates were obtained by centrifuging cells for 10 minutes at 14,000g under 4℃, then adding a loading buffer. The expression levels of ferroportin (FPN), transferrin, transferrin receptor, DMT1, and ferritin were determined by subjecting the cell lysates to electrophoresis on denaturing polyacrylamide gels (8-10%).

3. Result and discussion

3.1 Characterization of siramesine@Fe(III) PDA NPs

Dopamine tends self-polymerization under alkaline conditions, which is conducive to manufacturing PDA NPs. Thanks to the chelation affection of the catechol group and π-π stacking interactions of aromatic rings in the dopamine unit, PDA NPs display a high affinity for Fe^{3+} ions and siramesine. The siramesine@Fe(III) PDA NPs nano-complex was obtained by loading siramesine and Fe^{3+} ions onto PDA NPs by π-π superposition and chelation, respectively. Screening the dosage of siramesine/NPs with fixed Fe^{3+} ions concentration, with the increase in the NPs/siramesine weight ratio, the loading content of siramesine increased steadily. Since there were similar chemical properties, it can be seen from the literature[41] that when the feeding proportion is 2/0.8, the loading rate of NPs is higher and the particle size is suitable. Due to using similar synthetic means, the content of siramesine of NPs was determined by HPLC and LC was expected to be 38±1.3%. While an ICP-OES measured the content of iron, and then the LE was anticipated to be 18±1.3. The prospect EE of siramesine and $FeCl_3 \cdot 6H_2O$ were 71±1.1% and 27±1.6%, respectively. The high carrying ability of both siramesine and Fe(III) on PDA serves as evidence for the excellent drug delivery capabilities of PDA, highlighting its superiority as a platform for drug delivery. Based on FESEM analysis, the morphology of siramesine@Fe(III) nanoparticles closely resembled that of PDA nanoparticles. The predicted hydrodynamic dimensions and surface charge of the siramesine@Fe(III) nanoparticles were estimated to be

between 110-150 nm and 13-19 mV, respectively.

Due to maintaining a high metabolism level, cancer cells always generate an acid environment called the tumor microenvironment (TME). In addition, nanoparticles internalized by cells are always transported to lysosomes by the cell, which has a lower pH level environment than TME. Noteworthy, siramesine and Fe^{3+} ions' connection with PAD NPs are both acid-sensitive chemical linkage. NPs were dropped into different simulative environments, including the natural biological surroundings for pH 7.4, the TME for pH 6.5 and the environment inside the lysosome for pH 5.0 to investigate release profiles. According to the reference that used similar PDA NPs, the concentration of the drug released increases simultaneously as the pH value decreases. It was predicted from the reference[41] that in different acidic stimulation, the release level of siramesine from NPs increased by 15±0.4% at pH 6.5 compared to pH 7.4 and higher by 38±0.5 at pH 5.0. *In vitro* release contents of Fe from Fe(III)PP@SAS after incubation at different pH values via ICP-OES. With the upper pH value, the expected release efficiency of ferric ions is higher.

3.2 Cellular uptake and cytotoxicity

To illustrate the uptake of siramesine@Fe(III) PDA NPs by cells, MDA-MB-231 cells strained by DAPI were cultured with Fluorescein isothiocyanate (FITC)-labelled siramesine@Fe(III) PDA NPs and observed under the CLSM. Similar experiments in the references show the gradually enhanced green fluorescence intensity emitted by FITC can be observed around the blue fluorescence emitted by the DAPI-stained cell nucleus, which indicates that NPs successfully enter into the intracellular. And the ratio of siramesine@Fe(III) NPs internalization cells was expected to rise from 5±1.3% at one hour to 95±0.8% at 6h, which FCM detected. These prospective results demonstrated that NPs had good cell internalization and could be quickly and effectively internalized by cells.

The CCK-8 assay kit was applied to investigate the cells viability treated with siramesine@Fe(III) NPs to quantify their cytotoxic effects. We measured the percentage of cell death under the different contents of NPs (0, 5, 10, 20, 40, 80μg/mL), which displays apparent concentration-dependent cytotoxicity in MDA-MB-231 cells. Cell viability under the highest content siramesine@Fe(III) PDA NPs group anticipated to decrease by 25±1.8% compared with the control group. As a promising anti-cancer drug, drug-release affection under the TME was significant. Thus we used the excess H_2O_2 to emulate the tumour surroundings circumstances and investigated the cytotoxicity of NPs to specific MDA-MB-231 cells. Compared to cells dealt with H_2O_2 along without a significant decrease in cell viability being observed, H_2O_2 and NPs (concentration: 40μg/mL) co-treated cell viability expected to decrease to 40±1.4% at pH 6.5. To determine the synergistic effect of siramesine@Fe(III) PDA NPS, the toxicity of various treatments on MDA-MB-231 cells was studied. $FeCl_3$ group didn't observe significant cytotoxicity in cells. It was predicted that compared with 85±1.7% in the siramesine drug-alone group, cell viability was reduced by 19% to 66± 1.3 in the siramesine@Fe(III) PDA NPs, which showed more substantial toxicity to MDA-MB-231 cells. In addition, NPs groups displayed a more valid inhibition of cells than Fe(III)@PDA NPs. Cells were treated with calcein AM and PI to observe further the cell death caused by siramesine@Fe(III) PDA NPs. It was anticipated that compared with strong red fluorescence emitting from the treatment group of siramesine@Fe(III) PDA NPs, $FeCl_3$ and siramesine treated groups were surveyed with the strong green fluorescent emission. These results illustrated that the conjunctly use of siramesine and Fe^{3+} has a synergistic effect on the cytotoxicity of MDA-MB-231 cells.

3.3 The mechanism of siramesine@Fe(III) PDA NPs induce ferroptosis

One of the essential features of ferroptosis is abnormal ferric accumulation-induced LPO caused ROS generation to deadly cellular levels[12]. Thus, ROS is one of the most important hallmarks of ferroptosis detection. The creation of ROS was measured through the DCFH-DA probe. The cells group treated by the siramesine@Fe(III) PDA NPs illustrated a higher fluorescence with the DCFH-DA probe than those treated by $FeCl_3$ and siramesine, respectively. The above results demonstrate that ROS is involved in siramesine@Fe(III) PDA NPs-induced cell death.

Ferroptosis is an Fe-dependent nonapoptotic cell death; one essential characteristic is the abnormal intracellular ferric ions accumulation[11]. The content of ferric ions in cells is regarded as a vital indicator of whether cells can produce high levels of ROS through sufficient Fenton reaction to lead to iron death. And ferroportin (FPN) is the exclusive protein known to transport ferric ions from the intracellular to the extracellular. Under siramesine@Fe(III) PDA NPs-induced ferroptosis, siramesine inhibits the expression of FPN, inducing an obstacle to iron ions exportation, and NPs provide additional iron ions, thus predisposing to ferroptosis. The over capacity of cells with iron ions can trigger the Fenton reaction to boost sensitive tumour cells to ferroptosis. Therefore, the intracellular iron was measured by Prussian blue staining. Compared with $FeCl_3$ treating alone, the siramesine and Fe(III)@PDA NPs treatment group showed a more apparent blue stain. And siramesine@Fe(III) PDA NPs

group was observed to have the darkest blue dye than other groups. Iron Assay Kits-Colorimetric detected specific iron content, and similar to the above result, the $FeCl_3$ group merely showed a tiny difference compared with the control group. And siramesine@Fe(III) PDA NPs group detected the highest iron level. These results demonstrated that high Fe ions levels play an essential role in siramesine@Fe(III) PDA NPs.

Intracellular iron ions were regulated and controlled by many different relative proteins such as FPN, transferrin, ferritin, etc. Since FPN is the only iron extracellular transporter, excessive intracellular iron content leads to increased expression of compensatory FPN[26,33]. To investigate the mechanism of NPs-induced iron ions accumulation, we researched whether iron regulatory proteins are changed after the treatment of NPs. Collecting cell lysate after different treatments and western blotted for various iron ions transfer relative proteins. The expression of ferroportin (FPN) was expected to increase by treating with Fe(III)@PDA NPs in the MDA-MB-231 cells. In contrast, FPN expression increased by treating with siramesine or siramesine@Fe(III) PDA NPs. To ensure the effect leading to intracellular iron accumulation was not due to alters of other iron transport protein expression, the content of other relevant proteins such as transferrin receptor, DMT1 and ferritin were measured, and no significant changes were found after siramesine treatment.

4. Conclusion

In summary, based on the superiority of solid bioadhesion, drug loading capacity and excellent biocompatibility of PDA, we composed a new nanoplatform by loading ferric iron and siramesine on PDA, which shows a synergistic killing effect on breast cancer cells by ferroptosis and have a tremendous potent on cancer therapy. Affected by the acidic environment of the TME, the nanomaterial tends to decompose and release loaded ferric iron and siramesine. Upon entering the endocellular endosome, the ferric iron loaded onto the nanomaterial was discharged and converted to ferrous iron via metal reductase. This process initiated the Fenton reaction, which led to the generation of ·OH radicals and ultimately induced ferroptosis, leading to the destruction of cancer cells. The released siramesine inhibited FPN, the only transport protein responsible for removing iron ions from cells, can enhance iron accumulation in the cell and induce the ferroptosis caused by the Fenton reaction. Delivering iron ions to tumor cells and preventing tumor cells from transporting excess iron delivery out of the cells synergically enhanced the ferroptosis response in cancer cells, greatly enhancing the inhibitory effect of drugs on cancer cells. All in all, these findings indicated that ferric iron and siramesine-loaded nanoparticles (siramesine@Fe(III)PDA NPs) have an excellent potential for a significant cancer theranostic strategy.

References

[1] Waks, A. G.; Winer, E. P. Breast Cancer Treatment: A Review. JAMA 2019, 321 (3), 288–300. https://doi.org/10.1001/jama.2018.19323.

[2] Sung, H.; Ferlay, J.; Siegel, R. L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA. Cancer J. Clin. 2021, 71 (3), 209–249. https://doi.org/10.3322/caac.21660.

[3] Liu, J.; Shen, J.-X.; Wen, X.-F.; Guo, Y.-X.; Zhang, G.-J. Targeting Notch Degradation System Provides Promise for Breast Cancer Therapeutics. Crit. Rev. Oncol. Hematol. 2016, 104, 21–29. https://doi.org/10.1016/j.critrevonc.2016.05.010.

[4] Ono, M.; Ogilvie, J. M.; Wilson, J. S.; Green, H. J.; Chambers, S. K.; Ownsworth, T.; Shum, D. H. K. A Meta-Analysis of Cognitive Impairment and Decline Associated with Adjuvant Chemotherapy in Women with Breast Cancer. Front. Oncol. 2015, 5, 59. https://doi.org/10.3389/fonc.2015.00059.

[5] Shapiro, C. L.; Recht, A. Side Effects of Adjuvant Treatment of Breast Cancer. N. Engl. J. Med. 2001, 344 (26), 1997–2008. https://doi.org/10.1056/NEJM200106283442607.

[6] Saeki, T.; Tsuruo, T.; Sato, W.; Nishikawsa, K. Drug Resistance in Chemotherapy for Breast Cancer. Cancer Chemother. Pharmacol. 2005, 56 (1), 84–89. https://doi.org/10.1007/s00280-005-0106-4.

[7] Musgrove, E. A.; Sutherland, R. L. Biological Determinants of Endocrine Resistance in Breast Cancer. Nat. Rev. Cancer 2009, 9 (9), 631–643. https://doi.org/10.1038/nrc2713.

[8] El Ansari, R.; McIntyre, A.; Craze, M. L.; Ellis, I. O.; Rakha, E. A.; Green, A. R. Altered Glutamine Metabolism in Breast Cancer; Subtype Dependencies and Alternative Adaptations. Histopathology 2018, 72 (2), 183–190. https://doi.org/10.1111/his.13334.

[9] Carneiro, B. A.; El-Deiry, W. S. Targeting Apoptosis in Cancer Therapy. Nat. Rev. Clin. Oncol. 2020, 17 (7), 395–417. https://doi.org/10.1038/s41571-020-0341-y.

[10] Hanahan, D.; Weinberg, R. A. Hallmarks of Cancer: The next Generation. Cell 2011, 144 (5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013.

[11] Dixon, S. J.; Lemberg, K. M.; Lamprecht, M. R.; Skouta, R.; Zaitsev, E. M.; Gleason, C. E.; Patel, D. N.; Bauer, A. J.; Cantley, A. M.; Yang, W. S.; Morrison, B.; Stockwell, B. R. Ferroptosis: An Iron-Dependent Form of Nonapoptotic Cell Death. Cell 2012, 149 (5), 1060–1072. https://doi.org/10.1016/j.cell.2012.03.042.

[12] Stockwell, B. R.; Friedmann Angeli, J. P.; Bayir, H.; Bush, A.

I.; Conrad, M.; Dixon, S. J.; Fulda, S.; Gascón, S.; Hatzios, S. K.; Kagan, V. E.; Noel, K.; Jiang, X.; Linkermann, A.; Murphy, M. E.; Overholtzer, M.; Oyagi, A.; Pagnussat, G. C.; Park, J.; Ran, Q.; Rosenfeld, C. S.; Salnikow, K.; Tang, D.; Torti, F. M.; Torti, S. V.; Toyokuni, S.; Woerpel, K. A.; Zhang, D. D. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. Cell 2017, 171 (2), 273–285. https://doi.org/10.1016/j.cell.2017.09.021.

[13] Xie, Y.; Hou, W.; Song, X.; Yu, Y.; Huang, J.; Sun, X.; Kang, R.; Tang, D. Ferroptosis: Process and Function. Cell Death Differ. 2016, 23 (3), 369–379. https://doi.org/10.1038/cdd.2015.158.

[14] Yang, W. S.; SriRamaratnam, R.; Welsch, M. E.; Shimada, K.; Skouta, R.; Viswanathan, V. S.; Cheah, J. H.; Clemons, P. A.; Shamji, A. F.; Clish, C. B.; Brown, L. M.; Girotti, A. W.; Cornish, V. W.; Schreiber, S. L.; Stockwell, B. R. Regulation of Ferroptotic Cancer Cell Death by GPX4. Cell 2014, 156 (1), 317–331. https://doi.org/10.1016/j.cell.2013.12.010.

[15] Angeli, J. P. F.; Shah, R.; Pratt, D. A.; Conrad, M. Ferroptosis Inhibition: Mechanisms and Opportunities. Trends Pharmacol. Sci. 2017, 38 (5), 489–498. https://doi.org/10.1016/j.tips.2017.02.005.

[16] Banjac, A.; Perisic, T.; Sato, H.; Seiler, A.; Bannai, S.; Weiss, N.; Kölle, P.; Tschoep, K.; Issels, R. D.; Daniel, P. T.; Conrad, M.; Bornkamm, G. W. The Cystine/Cysteine Cycle: A Redox Cycle Regulating Susceptibility versus Resistance to Cell Death. Oncogene 2008, 27 (11), 1618–1628. https://doi.org/10.1038/sj.onc.1210796.

[17] Ray, P. D.; Huang, B.-W.; Tsuji, Y. Reactive Oxygen Species (ROS) Homeostasis and Redox Regulation in Cellular Signaling. Cell. Signal. 2012, 24 (5), 981–990. https://doi.org/10.1016/j.cellsig.2012.01.008.

[18] Bystrom, L. M.; Guzman, M. L.; Rivella, S. Iron and Reactive Oxygen Species: Friends or Foes of Cancer Cells? Antioxid. Redox Signal. 2014, 20 (12), 1917–1924. https://doi.org/10.1089/ars.2012.5014.

[19] Conrad, M.; Pratt, D. A. The Chemical Basis of Ferroptosis. Nat. Chem. Biol. 2019, 15 (12), 1137–1147. https://doi.org/10.1038/s41589-019-0408-1.

[20] Yang, W. S.; Stockwell, B. R. Ferroptosis: Death by Lipid Peroxidation. Trends Cell Biol. 2016, 26 (3), 165–176. https://doi.org/10.1016/j.tcb.2015.10.014.

[21] Hassannia, B.; Vandenabeele, P.; Vanden Berghe, T. Targeting Ferroptosis to Iron Out Cancer. Cancer Cell 2019, 35 (6), 830–849. https://doi.org/10.1016/j.ccell.2019.04.002.

[22] Dixon, S. J.; Stockwell, B. R. The Role of Iron and Reactive Oxygen Species in Cell Death. Nat. Chem. Biol. 2014, 10 (1), 9–17. https://doi.org/10.1038/nchembio.1416.

[23] Bogdan, A. R.; Miyazawa, M.; Hashimoto, K.; Tsuji, Y. Regulators of Iron Homeostasis: New Players in Metabolism, Cell Death, and Disease. Trends Biochem. Sci. 2016, 41 (3), 274–286. https://doi.org/10.1016/j.tibs.2015.11.012.

[24] Liu, X.-B.; Yang, F.; Haile, D. J. Functional Consequences of Ferroportin 1 Mutations. Blood Cells. Mol. Dis. 2005, 35 (1), 33–46. https://doi.org/10.1016/j.bcmd.2005.04.005.

[25] Nemeth, E.; Tuttle, M. S.; Powelson, J.; Vaughn, M. B.; Donovan, A.; Ward, D. M.; Ganz, T.; Kaplan, J. Hepcidin Regulates Cellular Iron Efflux by Binding to Ferroportin and Inducing Its Internalization. Science 2004, 306 (5704), 2090–2093. https://doi.org/10.1126/science.1104742.

[26] Donovan, A.; Lima, C. A.; Pinkus, J. L.; Pinkus, G. S.; Zon, L. I.; Robine, S.; Andrews, N. C. The Iron Exporter Ferroportin/Slc40a1 Is Essential for Iron Homeostasis. Cell Metab. 2005, 1 (3), 191–200. https://doi.org/10.1016/j.cmet.2005.01.003.

[27] De Domenico, I.; Ward, D. M.; Nemeth, E.; Vaughn, M. B.; Musci, G.; Ganz, T.; Kaplan, J. The Molecular Basis of Ferroportin-Linked Hemochromatosis. Proc. Natl. Acad. Sci. U. S. A. 2005, 102 (25), 8955–8960. https://doi.org/10.1073/pnas.0503804102.

[28] Schimanski, L. M.; Drakesmith, H.; Merryweather-Clarke, A. T.; Viprakasit, V.; Edwards, J. P.; Sweetland, E.; Bastin, J. M.; Cowley, D.; Chinthammitr, Y.; Robson, K. J. H.; Townsend, A. R. M. In Vitro Functional Analysis of Human Ferroportin (FPN) and Hemochromatosis-Associated FPN Mutations. Blood 2005, 105 (10), 4096–4102. https://doi.org/10.1182/blood-2004-11-4502.

[29] Pelucchi, S.; Mariani, R.; Salvioni, A.; Bonfadini, S.; Riva, A.; Bertola, F.; Trombini, P.; Piperno, A. Novel Mutations of the Ferroportin Gene (SLC40A1): Analysis of 56 Consecutive Patients with Unexplained Iron Overload. Clin. Genet. 2008, 73 (2), 171–178. https://doi.org/10.1111/j.1399-0004.2007.00950.x.

[30] Ma, S.; Dielschneider, R. F.; Henson, E. S.; Xiao, W.; Choquette, T. R.; Blankstein, A. R.; Chen, Y.; Gibson, S. B. Ferroptosis and Autophagy Induced Cell Death Occur Independently after Siramesine and Lapatinib Treatment in Breast Cancer Cells. PloS One 2017, 12 (8), e0182921. https://doi.org/10.1371/journal.pone.0182921.

[31] Geng, N.; Zhou, H. Knockdown of Ferroportin Accelerates Erastin-Induced Ferroptosis in Neuroblastoma Cells. 11.

[32] Namgaladze, D.; Fuhrmann, D. C.; Brüne, B. Interplay of Nrf2 and BACH1 in Inducing Ferroportin Expression and Enhancing Resistance of Human Macrophages towards Ferroptosis. Cell Death Discov. 2022, 8 (1), 327. https://doi.org/10.1038/s41420-022-01117-y.

[33] Laskar, A.; Ghosh, M.; Khattak, S. I.; Li, W.; Yuan, X.-M. Degradation of Superparamagnetic Iron Oxide Nanoparticle-Induced Ferritin by Lysosomal Cathepsins and Related Immune Response. Nanomed. 2012, 7 (5), 705–717. https://doi.org/10.2217/nnm.11.148.

[34] Boya, P.; Kroemer, G. Lysosomal Membrane Permeabilization in Cell Death. Oncogene 2008, 27 (50), 6434–6451. https://doi.org/10.1038/onc.2008.310.

[35] Ma, S.; Henson, E. S.; Chen, Y.; Gibson, S. B. Ferroptosis Is Induced Following Siramesine and Lapatinib Treatment of

Breast Cancer Cells. Cell Death Dis. 2016, 7, e2307. https://doi.org/10.1038/cddis.2016.208.
[36] Liu, M.; Liu, B.; Liu, Q.; Du, K.; Wang, Z.; He, N. Nanomaterial-Induced Ferroptosis for Cancer Specific Therapy. Coord. Chem. Rev. 2019, 382, 160–180. https://doi.org/10.1016/j.ccr.2018.12.015.
[37] Ma, P.; Xiao, H.; Yu, C.; Liu, J.; Cheng, Z.; Song, H.; Zhang, X.; Li, C.; Wang, J.; Gu, Z.; Lin, J. Enhanced Cisplatin Chemotherapy by Iron Oxide Nanocarrier-Mediated Generation of Highly Toxic Reactive Oxygen Species. Nano Lett. 2017, 17 (2), 928–937. https://doi.org/10.1021/acs.nanolett.6b04269.
[38] Liu, X.; Cao, J.; Li, H.; Li, J.; Jin, Q.; Ren, K.; Ji, J. Mussel-Inspired Polydopamine: A Biocompatible and Ultrastable Coating for Nanoparticles in Vivo. ACS Nano 2013, 7 (10), 9384–9395. https://doi.org/10.1021/nn404117j.
[39] Farokhi, M.; Mottaghitalab, F.; Saeb, M. R.; Thomas, S. Functionalized Theranostic Nanocarriers with Bio-Inspired Polydopamine for Tumor Imaging and Chemo-Photothermal Therapy. J. Controlled Release 2019, 309, 203–219. https://doi.org/10.1016/j.jconrel.2019.07.036.
[40] Li, H.; Yin, D.; Li, W.; Tang, Q.; Zou, L.; Peng, Q. Polydopamine-Based Nanomaterials and Their Potentials in Advanced Drug Delivery and Therapy. Colloids Surf. B Biointerfaces 2021, 199, 111502. https://doi.org/10.1016/j.colsurfb.2020.111502.