



Reactive oxygen species mediated-photo-sono-ablation of melanoma cells using a novel nanocomposite of platinum-polypyrrole as a nanozyme

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ARTICLE INFO

Keywords:

Near-infrared
Laser therapy
Therapeutic ultrasound
Conductive polymer
Pyrrole

ABSTRACT

Tumor eradication in non-invasive photothermal (PTT) and sonodynamic therapy (SDT) approaches is favorable in melanoma cancer. However, finding a sensitizer for synchronous PTT and SDT is a desirable request, especially if this sensitizer material has other benefits in tumor treatment. Here, a platinum (Pt)/polypyrrole nanocomposite (Pt@PPy NC) was introduced for synchronous synergistic PTT and SDT of melanoma cancer cells. To compare and clarify the effect of the nanocomposite components, platinum nanoparticles (NPs) and polypyrrole (PPy) NPs were also synthesized and evaluated. Pt@PPy NC consisted of near-spherical Pt NPs adhered by a network of PPy. Pt NPs, PPy NPs, and Pt@PPy NC had mean diameters of 13 ± 7 , 177 ± 48 , and 16.5 ± 2.5 nm, respectively. The results of cytotoxicity assessment of B16-F10 cells treated with Pt@PPy NC revealed that while it was cytocompatible, it represented enhanced photothermal and sonodynamic cytotoxicity (with a deeper cytotoxicity effect, compared to Pt NPs and PPy NPs). Cell viability assessments revealed that the cell killing ability was attained in a synergistic manner from the effects of Pt@PPy NC, 808-nm light, and ultrasound waves. Detection of intracellular reactive oxygen species (ROS) production revealed that the effect of PTT and SDT using Pt@PPy NC arose from induction of oxidative stress. Pt@PPy NC also represented catalase-mimicking activity and could be considered for combination therapy.

1. Introduction

Melanoma is the deadliest type of skin cancer, related to melanocytes, and the most aggressive type of skin cancer (Domingues et al., 2018). In 2023, it is predicted that 97,610 new patients will be diagnosed with melanoma in the United States alone; approximately 7990 cases will result in death (Siegel et al., 2023). Different treatment modalities can be applied according to the situation of melanoma tumors, including stage, location, and genetic profile (Domingues et al., 2018). Surgery for early-stage melanoma and non-surgical therapies for advanced melanoma including chemotherapy, radiotherapy, and immunotherapy may be usual approaches for melanoma patients. However, melanoma is one of the most difficult cancers to cure (Arai et al., 2018). Typical cancer treatments, including surgery and radiotherapy have side effects, low patient satisfaction, and less precision

(Haume et al., 2016; Wyld et al., 2015), chemotherapy drugs have the potential to damage normal cells and healthy tissues, and metastatic melanomas are often resistant to anti-cancer drugs. Therefore, development of treatments with minimized adverse impacts and maximized benefits are highly interested (Wyld et al., 2015; Moran, 2015).

Phototherapy is a novel, unconventional, and non-invasive cancer treatment. This modality includes two common approaches which are photodynamic therapy (PDT) and photothermal therapy (PTT) (Shibu et al., 2013; Bobrovnikova-Marjon and Hurov, 2014). PTT generates sufficient heat for thermal ablation upon near-infrared (NIR) laser radiation of photothermal agents (PA) (Reinisch, 1996; Kim and Lee, 2018). The maximized invasiveness, ease of implementation, and manageability of PTT, which spares blood cells and soft tissues from the harm of light irradiation, have made PTT a promising method for cancer treatment (Liang et al., 2020).

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Sonodynamic therapy (SDT) is known as a minimally invasive, non-ionizing, and inexpensive treatment for cancer using low-intensity ultrasound (US) waves and non-toxic sonosensitizers (Yu et al., 2004). In SDT, in addition to being safer due to its non-radiative nature, the problem of light penetration into deep tissues and tumors is overcome by increasing the radiation permeability (Tachibana et al., 2008). US waves cause various thermal and non-thermal interactions with the body tissues including interactions of sonosensitizers with an acoustic field which causes generation of reactive oxygen species (ROS) and induces toxic effects on the cells and eventually leads to tumor eradication (Haghighi et al., 2024; Rosenthal et al., 2004a).

In PTT and SDT, noble metal nanoparticles are utilized as photo/sonosensitizers. Gold nanoparticles (AuNPs) with a variety of coating materials, size, conjugation, and shape are utilized as photo/sonosensitizers (Kayani et al., 2024). Platinum (Pt) nanostructures are thermally stable and have surface modification ability, inherent anticancer capacity, drug transport capability, a high NIR light absorption efficiency and biocompatibility (Puja and Kumar, 2019; Gutiérrez de la Rosa et al., 2022). These characteristics cause them to be suitable for cellular absorption and tumor growth inhibition (Zhu et al., 2017), and desirable applications of sensing, sensitizing (for light, and gamma- and X-ray (Negahdary and Heli, 2018; Daneshvar et al., 2020; Salehi et al., 2019; Li et al., 2017)), delivering and bacteria killing in nanomedicine (Puja and Kumar, 2019; Gutiérrez de la Rosa et al., 2022) including PTT and PDT.

Up to now, organic polymers including polypyrrole (PPy) have been applied as photo-sonotherapy (PST) agents due to their strong NIR light absorbance, low cytotoxicity, high conductivity, and biocompatibility (Geetha et al., 2006; Pishahang et al., 2018; Heli and Yadegari, 2014). Additionally, PPy nanostructures have been utilized in nerve regeneration, biosensor fabrication, and drug delivery (Ateh et al., 2006). On the other hand, coating NPs with organic polymers has resulted in reduced cytotoxicity, increased biocompatibility, higher photothermal efficiency, excellent photostability, and higher chemical stability (Hou et al., 2015; Xiao et al., 2016).

So far, numerous composite structures with PPy coating/loading have been investigated as promising sensitizers in PST including Fe-Pt NPs (Phan et al., 2017), carbon nanotubes (Behzadpour et al., 2019), an iridium complex (Xue et al., 2016), palladium NPs (Liu et al., 2016), gold nanorods (Du et al., 2015), silica fibers (Merlini et al., 2012), and TiO₂ (He et al., 2019). The stabilization of gold NPs was improved through the PPy coating which maintained the spectral characteristics and prevented aggregation (Lin et al., 2014). Incorporation of a thin layer of PPy as a shell on TiO₂ nanofibers led to improved photocatalytic performance via effectively narrowing the band gap and enhancing the quantum yield (Yao et al., 2016). Coating of Fe-Pt NPs with PPy resulted in improved photothermal stability and higher NIR absorbance that induced cytocompatibility and cancer cell killing upon laser irradiation (Phan et al., 2017). It is noteworthy that, to the best of our knowledge, the properties of Pt/polypyrrole nanocomposite (Pt@PPy NC) as a sonosensitizer have yet to be explored. In Pt@PPy NC, PPy is polymerized in the presence of platinum salt within an SDS-stabilized medium, and forms a PPy coating around Pt NPs. This structure confers several advantages including strong absorption in NIR region and efficient photothermal conversion, excellent sonosensitizing effect, enhanced biocompatibility, improved stability and prevent aggregation during storage. Also, the conjugated aromatic backbone of PPy can promote noncovalent interaction (π - π stacking) with aromatic drugs or organic molecules, providing opportunities for drug loading and targeting.

In the present study, Pt NPs, PPy NPs, and a Pt@PPy NC were synthesized, their potency as photosensitizers working at 808-nm light, sonosensitizers for US exposure, synchronous light irradiation and US exposure, and their potential to generate ROS were evaluated. Subsequently, Pt@PPy NC was evaluated as a dual sensitizer in synchronous SDT and PTT for annihilating melanoma cancer in vitro.

2. Materials and methods

2.1. Materials

All chemicals were procured from Merck (Germany), Sigma (USA), or Scharlau (Spain). They were employed without any pre-purification. Deionized (DI) water was utilized throughout the experiments.

2.2. Synthesis of Pt NPs, PPy NPs, and Pt@PPy NC

To synthesize Pt NPs, we prepared 15.0 mL aqueous solution including 10.0 mmol L⁻¹ sodium dodecyl sulfate (SDS), 1.75 mol L⁻¹ formic acid and 1.0 mmol L⁻¹ H₂PtCl₆. The demanded quantity of the mentioned stock solutions was mixed in a Teflon beaker. Afterward, the blend was heated at 40 °C while being constantly stirred at a rotation speed of 500 rpm for 3 h. Then, the sediment was separated using a centrifuge, and it was rinsed with DI water. Eventually, the sediment was dried at ambient temperature.

To synthesize PPy NPs, we mixed 4.0 mmol L⁻¹ pyrrole (Py) with 4.0 mL of 1.0 mol L⁻¹ HCl at ambient temperature for 30 min to obtain a Py-HCl solution. Then, 200 μ L of 29% H₂O₂ was added to the mixture. The mixture was stirred until the reaction mixture color gently changed due to the Py polymerization. The solution was then left at room temperature for 12 h for polymerization completion. Next, the mixture was frequently separated and washed with DI water and methyl alcohol. Eventually, the filtrate was racked up and dried at 50 °C during 12 h.

To synthesize Pt@PPy NC, we added 10.0 μ L of Py to 5.0 mL of SDS aqueous solution (8.27 mmol L⁻¹) and then mixed them. To gain a uniform dispersion, the solution was stirred for 2 h. In the next step, 1.0 mL of H₂PtCl₆ aqueous solution (10.0 mmol L⁻¹) was added. The obtained mixture was then stored at 50 °C under stirring for 24 h. Finally, the product was repeatedly washed with water and ethanol to extract residual Py and dried under vacuum for 24 h.

2.3. Characterization of Pt NPs, PPy NPs, and Pt@PPy NC

UV-vis absorption spectra of Pt NPs, PPy NPs, and Pt@PPy NC were recorded individually using a Rayleigh double-beam spectrophotometer (China). The morphology and size of Pt NPs, PPy NPs, and Pt@PPy NC were determined using a TESCAN Mira 3-XMU field emission scanning electron microscope (Czech Republic). Molecular composition of PPy NPs and Pt@PPy NC was analyzed by a Fourier-transform infrared (FTIR) spectrometer of Bruker Tensor 27 (Germany). CHN elemental analysis was performed to determine the carbon content of Pt@PPy NC by an ECS 4010 elemental analyzer (Costech, Italy).

2.4. Cell line preparation

B16-F10 (B16-F10) melanoma cells and HDF normal human dermal fibroblast cells derived from murine malignant melanoma was obtained from Pasteur Institute (Iran). B16-F10 and HDF were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin, 10,000 μ g mL⁻¹) from Gibco (USA) at 37 °C in a humidified atmosphere with 5% CO₂ in a cell culture incubator.

2.5. B16-F10 and HDF cell toxicity assessment in the presence of Pt NPs, PPy NPs, and Pt@PPy NC

B16-F10 and HDF cells were cultured in 96-well culture plates (1.0 $\times$ 10⁴ cells well⁻¹) and incubated for 24 h to stick to the wells. Next, the cells were treated with different concentrations (0 to 250 μ g mL⁻¹) of Pt NPs, PPy NPs, and Pt@PPy NC. In the next 24 h, the individual cytotoxic effects of Pt NPs, PPy NPs, and Pt@PPy NC were measured by the MTT assay. The optical absorbance of the wells was measured at 570 nm using a Stat Fax microplate reader (USA). The untreated cells were selected as

controls for each measurement. Cell viability was reported by calculating the ratio of absorbance per well to that of the control. All measurements were performed in triplicate for each concentration.

2.6. Diode laser and ultrasound instrumentations

A 808-nm diode laser from Thorlabs (Germany) equipped with a benchtop temperature controller was utilized. The optical output power density of the diode laser was fixed at 1.0 W cm^{-2} because of altering the spot size of the output mounting lens point and the distance between the lens and the target. For PTT, irradiation was accomplished on 96-well cell culture plates for 10 min.

A 215X ultrasonic apparatus from Novinmed (Iran) was utilized for US exposure. The instrument was equipped with an unfocused planar US transducer having a diameter of 4.0 cm, operating at a frequency of 1.0 MHz, an output intensity of 1.0 W cm^{-2} , and a duty ratio of 100%. For SDT, the center of the transducer was positioned directly under the center of each well with a specific concentration in a 96-well cell culture. The bottoms of the exposing wells were covered with ultrasonic gel, and the US exposure was carried out for 1 min.

2.7. Photothermal conversion efficiency (η) of Pt@PPy NC

η for Pt@PPy NC was determined using a time constant method. Samples of Pt@PPy NC ($50, 100$ and $250 \mu\text{g mL}^{-1}$) prepared in DI water in a glass cuvette were irradiated at 1.0 W cm^{-2} of 808-nm laser light. Temperature was monitored with a thermo-probe from Lutron (Taiwan) with a 0.01°C resolution. Once the temperature reached a steady state value, the laser was turned off, and the cuvette was left to cool naturally to reach the initial temperature.

2.8. Separate and synchronized photothermal and sonodynamic effect of Pt NPs, PPy NPs, and Pt@PPy NC on B16-F10 cells

24 h post-seeding, in vitro photothermal and sonodynamic effects of Pt NPs, PPy NPs, and Pt@PPy NC on B16-F10 cells were traced by the MTT assay. The cells for each condition were split into two groups of L^- (without laser irradiation) and L^+ (with laser irradiation) for photothermal evaluation, and U^- (without US exposure) and U^+ (with US exposure) for sonodynamic evaluation. For L^+ groups, the cells were incubated after being treated with 0 (control) and $100 \mu\text{g mL}^{-1}$ of Pt NPs, PPy NPs, and Pt@PPy NC separately for 4 h. For U^+ groups, the cells were incubated after being treated with 0 (control) and $100 \mu\text{g mL}^{-1}$ of Pt NPs, PPy NPs, and Pt@PPy NC separately for 4 h. In the next step, treatment was accomplished by laser irradiation at 1.0 W cm^{-2} for 10 min or US exposure at a power density of 1.0 W cm^{-2} for 1 min at 100% duty ratio of a frequency of 1.0 MHz. For L^- and U^- groups, the cells were incubated after being treated with 0 (control) and $100 \mu\text{g mL}^{-1}$ of Pt NPs, PPy NPs, and Pt@PPy NC separately for 4 h in the absence of any laser irradiation or US exposure. Afterward, the cell culture plates were incubated for 24 h at 37°C and 5% CO_2 in the cell incubator. Cell viability was then measured by the MTT assay by measuring the optical intensity of each well relative to the control well at 570 nm using a Stat Fax microplate reader (USA).

For synchronized evaluation of the photothermal and sonodynamic effects, the cells were separated into two groups of L^-U^- (without laser irradiation and US exposure) and L^+U^+ (with laser irradiation and US exposure). For L^+U^+ groups, the cells were incubated after being treated with $100 \mu\text{g mL}^{-1}$ of Pt NPs, PPy NPs, and Pt@PPy NC separately for 4 h. In the next step, the cells were firstly irradiated upon laser irradiation at a power density of 1.0 W cm^{-2} for 10 min, then immediately treated by US exposure at a power density of 1.0 W cm^{-2} for 1 min at 100% duty ratio and a frequency of 1.0 MHz. For L^-U^- groups, the cells were incubated after being treated with $100 \mu\text{g mL}^{-1}$ of Pt NPs, PPy NPs, and Pt@PPy NC separately for 4 h without laser irradiation and US exposure. Afterward, the cell culture plates were incubated for 24 h at 37°C with

5% CO_2 in the cell incubator. Cell viability was then measured by the MTT assay.

Cell viability was then measured by measuring the optical intensity of each well relative to the control well at 570 nm using a Stat Fax microplate reader (USA). Combination indices (CIs) were calculated using the results of B16-F10 cells viabilities to obtain conceivable synergistic effects in PTT (808-nm laser light irradiation along with individual effects of Pt NPs, PPy NPs, and Pt@PPy NC), SDT (US exposure along with individual effects of Pt NPs, PPy NPs, and Pt@PPy NC), and PST (laser light irradiation and US exposure synchronously along with individual effects of Pt NPs, PPy NPs, and Pt@PPy NC). When two agents, A and B, are defined as individually or synchronously affecting cell viability, CI is (Yao et al., 2016):

$$\text{CI} = V_{\text{I}} \times V_{\text{II}} / V_{\text{I-II}} \quad (1)$$

where, V_{I} , V_{II} , and $V_{\text{I-II}}$ are the cell viabilities attained after affections of agents A, B and synchronous A and B. Values of CIs can be > 1 , $= 1$, or < 1 , indicating domination of synergism, additive, or antagonism effects, respectively, of the two agents I and II.

2.9. Cell-free ROS production by Pt NPs, PPy NPs, and Pt@PPy NC upon US exposure

2',7'-dichlorofluorescein (DCF) was used to evaluate the intrinsic capacity of Pt NPs, PPy NPs, and Pt@PPy NC in ROS production upon US exposure. For this purpose, 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) was converted to 2',7'-dichlorodihydrofluorescein (H2DCF) by de-acetylation, and the fluorescence intensity was measured based on the previous procedure (Zahraie et al., 2023). An equal amount of the H2DCF solution was added to Pt NPs, PPy NPs, and Pt@PPy NC, followed by US exposure, and after incubation for 1 h, emission of fluorescence at 520 nm was measured after excitation at 485 nm using a microplate reader of Biotek (USA) using black plates. A solution of 100 mmol L^{-1} phosphate-buffered saline (PBS) was considered as a control.

2.10. Evaluation of intracellular ROS level

B16-F10 cells (1.0×10^4 cells well^{-1}) were seeded in 96-well plates, and treated as mentioned in section 2.7., except that $100 \mu\text{L}$ of a fresh H2DCF-DA solution ($50 \mu\text{mol L}^{-1}$) was added to each well 30 min before irradiation. Intracellular H2DCF-DA was de-esterified by endogenous esterases, oxidized by intracellular ROS and converted to the fluorescent compound DCF. Extracellular H2DCF-DA was removed from the cells by washing them three times with PBS. After that, $100 \mu\text{L}$ of 150 mmol L^{-1} NaCl, 0.1% Triton X-100, 50 mmol L^{-1} Tris-HCl, pH 8.0 (lysis buffer) was added to each well, and after 30 min, the fluorescence intensity at 520 nm was measured upon excitation at 485 nm, using a microplate reader (Biotek, Winooski, VT, USA) which was considered as a sign of intracellular ROS.

In addition, images from intracellular ROS generation in cell groups were recorded using an Olympus BX61 fluorescence microscope (Japan).

2.11. Catalase activity detection of Pt@PPy NC

To evaluate the inherent capacity of Pt@PPy NC to induce catalase mimetic activity, absorbance measurements were conducted using UV-vis spectrophotometry at 240 nm (Beers and Sizer, 1952). Catalase activity was assessed by incubating 0.75 mL of Pt@PPy NC ($100 \mu\text{g mL}^{-1}$) or PBS (as control) with an equal amount of hydrogen peroxide (30 mmol mL^{-1}) at 25°C . The absorbance spectra (200–700 nm) were recorded at time zero and after 180 min. Catalase activity was measured as degradation rate of hydrogen peroxide at 240 nm (Beers and Sizer, 1952) as:

$$\text{Degradation ratio\%} = [(C_0 - C) / C_0] \times 100\% \quad (2)$$

where C_0 is H_2O_2 initial concentration of and C is the H_2O_2 concentration after 180 min. Molar extinction coefficient of H_2O_2 at 240 nm was considered $43.6 \text{ M}^{-1} \text{ cm}^{-1}$.

2.12. Statistics

At least three parallel measurements were executed to measure each quantity. One-way ANOVA with a Tukey post hoc and independent t -test was performed to analyze the statistical significance of the values using SPSS software. P-values less than 0.05 were considered statistically significant.

3. Results and discussion

The superior characteristics of Pt NPs make them the focus of nanomedicine investigations. Besides their desirable compatibility, elevated mass/surface ratio, compact size, enhanced reactivity, and electrocatalytic characteristics, their nanoenzyme properties that mimic superoxide dismutase and catalase-like activity (Pawar et al., 2021) make Pt NPs an ideal adjuvant candidate for cancer treatment. It is expected that the optical surface plasmon resonance properties and other characteristics of Pt NPs can be enhanced by selecting PPy as a suitable coating, making them a promising candidate for PST. Fig. 1 schematically shows the synthesis procedures of Pt NPs (A), PPy NPs (B), and Pt@PPy NC (C). PPy coating can stabilize Pt NPs and modify the surface to immobilize various biomolecules.

Fig. 2 presents field-emission scanning electron microscopy (FESEM) images of Pt NPs (A), PPy NPs (B), and Pt@PPy NC (C) at two distinct magnifications (a, b). Both Pt NPs and PPy NPs comprised near spherical

particles with some adherence. The average diameters of Pt NPs, and PPy NPs are 13 ± 7 and 177 ± 48 nm ($n = 50$), respectively. The Pt@PPy NC images indicated that the nanocomposite comprised particles of 16.5 ± 2.5 nm ($n = 50$) in diameter with rough surfaces and consists of Pt NPs adhered by a network of PPy. For the very different sizes and morphologies observed in the FESEM images, it should be mentioned that the synthesis mechanism of Pt NPs, PPy NPs, and Pt@PPy NC is very different. While during the synthesis of Pt NPs the Pt precursor (H_2PtCl_6) is reduced by formic acid and SDS acts as a stabilizing agent, during the synthesis of Pt@PPy NC the Pt precursor is reduced by Py, and the resultant Pt NPs act as seeds for formation of PPy. On the other hand, during the synthesis of Pt@PPy NC, oxidative polymerization of Py is performed by the Pt precursor. Formation of PPy NPs, however, is performed by oxidative polymerization of Py by hydrogen peroxide in the presence of SDS stabilizer. Therefore, synthesis of Pt NPs, PPy NPs, and Pt@PPy NC go through quite different trajectories, and it is impossible to follow a similar route of synthesis for these materials (to obtain nanostructures of the same size and morphology).

Supplementary material S1 shows FTIR spectra for PPy NPs and Pt@PPy NC. PPy NPs showed a broad absorption band at around 3416 cm^{-1} attributing to N-H bond stretching. A peak at 1708 cm^{-1} corresponds to the C=C ring stretching vibrations (Ovando-Medina et al., 2011), while 1456 cm^{-1} band is assigned to C-N/C-C stretching modes within the pyrrole rings (Li et al., 2008). The peak at 1397 cm^{-1} arises from =C-H in-plane bending vibrations (Li et al., 2009). Additionally, bands around 1390 cm^{-1} and 780 cm^{-1} shared by both PPy NPs and Pt@PPy NC are attributed to C-H and N-H in-plane deformation (Hidayah et al., 2025). Pt@PPy NC spectrum exhibited a peak at 3433 cm^{-1} corresponding to hydroxyl groups (O-H) stretching vibrations, likely due to the adsorbed water. Two peaks at 795 and 1036 cm^{-1} are attributed to C-H wagging modes. The spectrum also features C=C

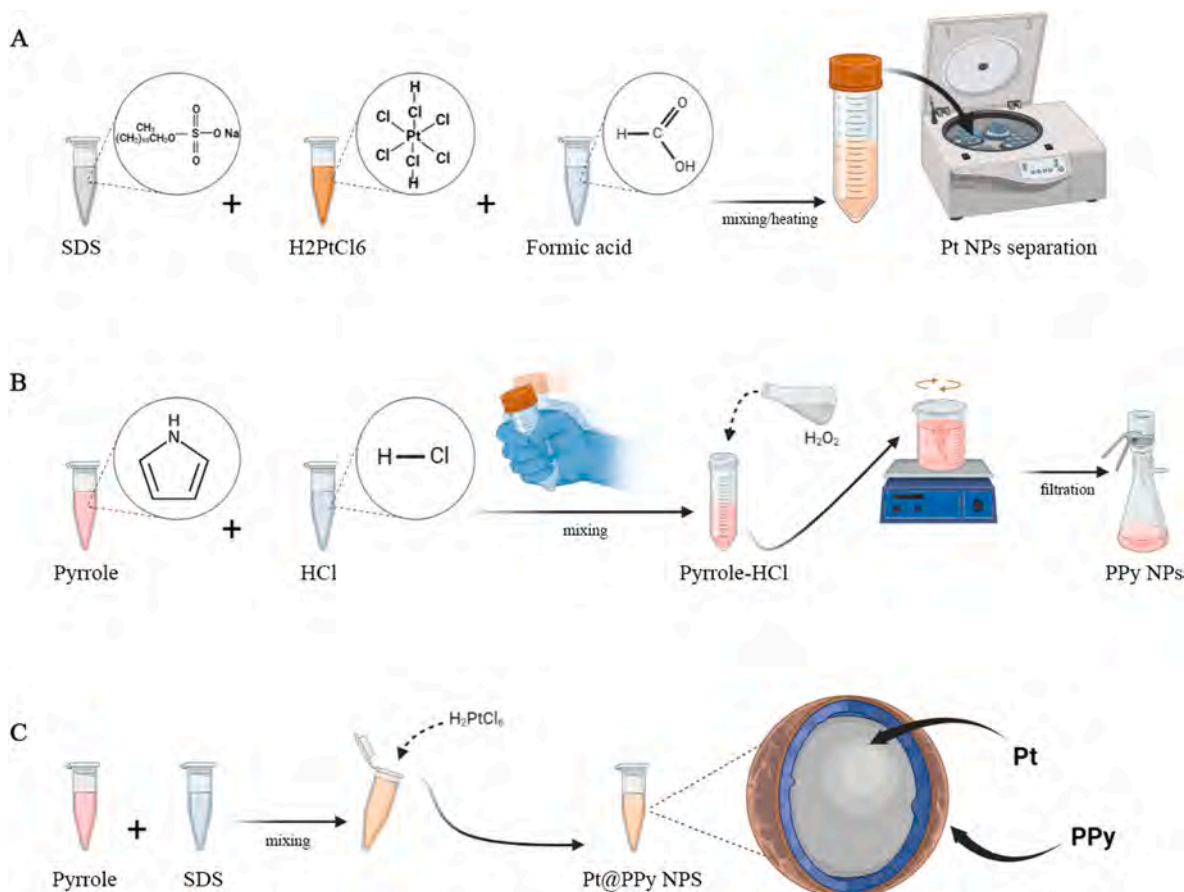


Fig. 1. A schematic presentation of the synthesis procedure of Pt NPs (A), PPy NPs (B), and Pt@PPy NC (C).

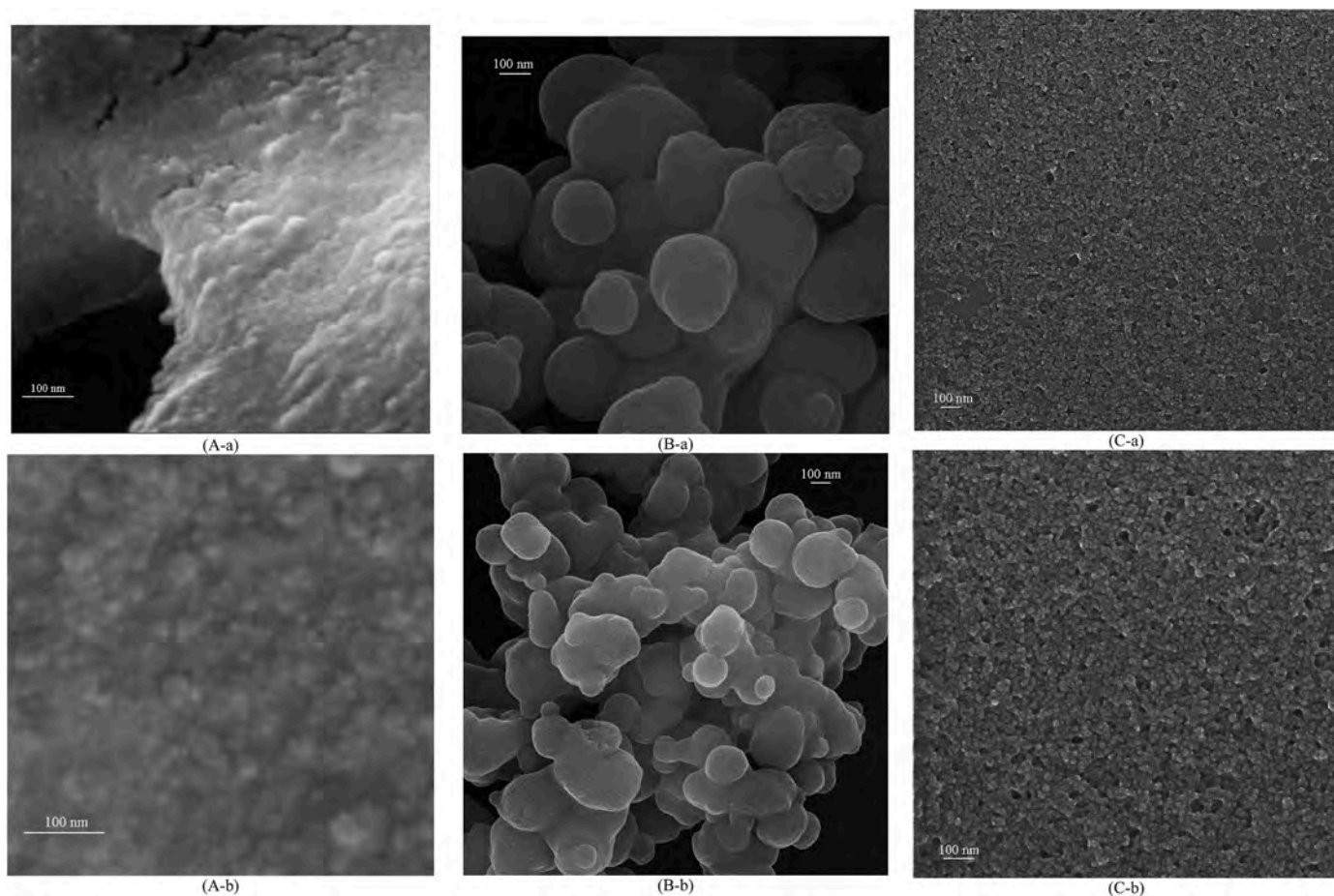


Fig. 2. FESEM images of Pt NPs (A), PPy NPs (B) and Pt@PPy NC (C).

stretching at 1391 and 1495 cm^{-1} , and two distinct peaks at 1620 and 1258 cm^{-1} , corresponding to C=N and C-N bonds, respectively (Lazar et al., 2025).

Supplementary material S2 shows a CHN chromatogram of Pt@PPy NC. The chromatogram resulted in C, H and N percents in Pt@PPy NC as 56.1, 5.3 and 16.3%, respectively. Therefore, Pt@PPy NC comprised 77.7 and 22.3% of PPy and Pt, respectively.

UV-vis absorbance profiles of Pt NPs, PPy NPs, and Pt@PPy NC are presented in Fig. 3. The spectra show that at 808 nm , PPy NPs exhibited the lowest and Pt@PPy NC exhibited the highest light absorption, indicating that Pt@PPy NC is an excellent NIR photoabsorber

nanomaterial applicable in PTT. The characteristics of Pt@PPy NC validate the successful synthesis of a suitable and promising nanomaterial with potential applications in PTT. Studies have also confirmed the effectiveness of PPy covers in different nanosystems that induce better hydrophilicity and colloidal stability (Han and Armes, 2003).

η for Pt@PPy NC was measured using the time-constant method and the following relations:

$$h \times S = m \times C_p / \tau \quad (3)$$

$$\eta = h \times S (T_E - T_A) - Q_D / (I \times (1 - 10^{-A})) \quad (4)$$

where h is the heat transfer coefficient, S is the exposed surface area, T_E and T_A are the equilibrium and ambient temperatures, Q_D is the heat dissipated by the cuvette containing DI water alone (measured independently as 7.1 mW under 808-nm irradiation), I is the incident laser power, and A is the sample absorbance at 808 nm for Pt@PPy NC of 50, 100 and $250\text{ }\mu\text{g mL}^{-1}$. The product $h \times S$ was obtained from the cooling time constant, τ , which was extracted from the cooling curve using the dimensionless temperature:

$$\theta = (T - T_A) / (T_E - T_A) \quad (5)$$

and,

$$t = -\tau \times \ln(\theta) \quad (6)$$

Temperature profiles under 808-nm irradiation of Pt@PPy NC suspensions (50 , 100 and $250\text{ }\mu\text{g mL}^{-1}$) are provided in supplementary material S3. Samples were irradiated until thermal equilibrium, and then allowed to cool naturally. Linear fits of $\ln(\theta)$ versus time (supplementary material S3) with the cooling data yielded the system

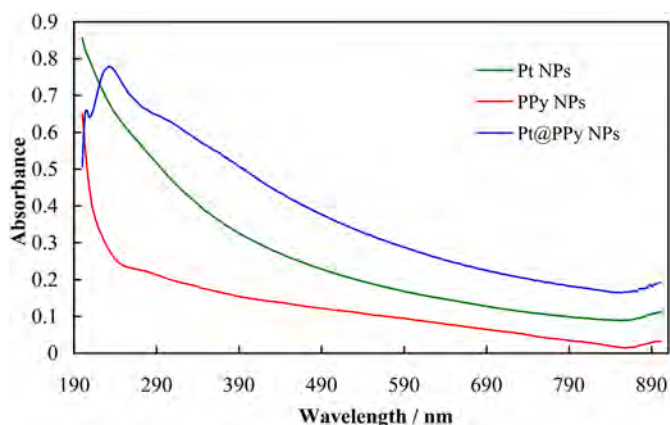


Fig. 3. Typical UV-vis absorption spectra of as-synthesized Pt NPs (A), PPy NPs (B) and Pt@PPy NC (C).

time constant τ , and thus the heat-transfer coefficient ($h \times S$) was obtained. Substituting ($h \times S$) and the measured absorbance at 808 nm into equation (4) gave η s of 62.5%, 74.7% and 94.2% for Pt@PPy NC at 50, 100 and 250 $\mu\text{g mL}^{-1}$, respectively. These results demonstrate the excellent and concentration-dependent photosensitizing ability of Pt@PPy NC. Irradiation of Pt@PPy NC at 250 $\mu\text{g mL}^{-1}$ with the highest η attained produced very high equilibrium temperatures in vitro that could cause high thermal damage. Irradiation of 100 $\mu\text{g mL}^{-1}$ with a substantially elevated photothermal efficiency (74.7%) led to a moderate temperature elevation (9 °C) sufficient to induce therapeutic hyperthermia without causing excessive overheating under irradiation conditions. Moderate hyperthermia (typically 42–48 °C) is often preferable in combined photothermal/sonodynamic protocols because it promotes tumor cell sensitization and can enhance ROS generation from sonosensitizers, while minimizing collateral damage and inflammatory responses associated with higher temperatures. Furthermore, a moderate temperature rise is more compatible with potential in vivo application where heat dissipation and tissue heterogeneity make tight temperature control critical. However, selection of an optimal concentration for therapeutic application requires balancing photothermal efficiency with biocompatibility and clinical practicality.

To assess the biocompatibility of Pt NPs, PPy NPs, and Pt@PPy NC, we investigated their effects on normal human dermal fibroblast (HDF) cells using the MTT assay following 24-h treatment with 10, 50, and 100 $\mu\text{g mL}^{-1}$. The obtained results (supplementary material S4) indicated that HDF cells remained viability at all the concentrations of Pt NPs, PPy NPs, and Pt@PPy NC, indicating minimal cytotoxicity of these materials toward the normal cells. Furthermore, Pt@PPy NC represented the highest biocompatibility, compared to Pt NPs and PPy NPs. In Pt@PPy NC, coating Pt NPs with PPy reduced toxicity, provided a favorable therapeutic index, and provided preliminary evidence of their biosafety for translational development. Previous studies has support these finding (Sun et al., 2016) with showing the biocompatibility of PPy for cell adhesion and proliferation of mesenchymal stem and osteoblastic cells (Lakard et al., 2009; Vaitkuviene et al., 2014).

Cytotoxicity of Pt NPs, PPy NPs, and Pt@PPy NC was evaluated against the B16-F10 melanoma cell line using the MTT cell proliferation assay. Fig. 4 illustrates the cell viability upon exposure to concentrations of Pt NPs, PPy NPs, and Pt@PPy NC. All the nanostructures exhibited biocompatibility up to 100 $\mu\text{g mL}^{-1}$, where the cell viability remained above 73%. However, at a concentration as high as 250 $\mu\text{g mL}^{-1}$, Pt NPs, PPy NPs, and Pt@PPy NC demonstrated mild toxicity with reduced cell

viabilities of 65, 71 and 64%, respectively. Based on the results of temperature elevation measurements and cytotoxicity toward HDF and B16-F10 cells, 100 $\mu\text{g mL}^{-1}$ of Pt@PPy NC represented a balanced compromise between high photothermal performance and biocompatibility considerations, making it an appropriate choice for our combined photothermal and sonodynamic experiments.

A literature review of the cytotoxic behavior of various synthesized Pt NPs indicates that Pt NPs and Pt@PPy NC synthesized in this study represented more cytocompatibility and reliability toward cellular interactions. In other words, a viability of >50% was attained even in the presence of 250 $\mu\text{g mL}^{-1}$ Pt NPs and Pt@PPy NC. However, Pt NPs capped with polyphenols from peppermint leaf extract exhibited cytotoxicity against HCT 116 human colorectal cancer cells with IC₅₀ of 20 $\mu\text{g mL}^{-1}$ (Yang et al., 2017). Another study showed that Pt/MgO NPs with a cuboid shape and 30–50 nm size were relatively innocuous to normal cells of human lung and colonic fibroblast cells, and toxic to human lung (A549) and colon (HT29) cancer cells, with HT29 cells being the most sensitive with IC₅₀ of 13.7 $\mu\text{g mL}^{-1}$ (Han and Armes, 2003). Pt/MgO NPs induced the production of ROS, decreased cellular glutathione levels, and increased lipid peroxidation. They also change the expression of Bcl-2 by downregulating it, and upregulate Bax and p53 tumor suppressor proteins in these cancer cells (Al-Fahdawi et al., 2021).

Pt NPs have been introduced as a promising nanomaterial for cancer therapy which can enter the tumor cells by passive diffusion and induce toxicity depending on the size, concentration, and exposure time. This toxicity results from the introduction of breaks in the DNA strands, which inhibits replication and leads to cell growth arrest and apoptosis (Porcel et al., 2010; Gehrke et al., 2011). Another way that Pt NPs affect cells is through inhibition of metabolic activity, generating hydroxyl radicals, and releasing active Pt^{2+} ions; the later way as a strategy is currently used in radiotherapy (Borowik et al., 2019). It has also been reported that DNA damage is increased in human cells exposed to Pt NPs capped with polyvinyl alcohol, inducing cell accumulation in the S phase of the cell cycle and undergoing apoptosis. Following Pt NP treatment, p53 upregulation, p53 and p21 phosphorylation, along with proliferating cell nuclear antigen downregulation were observed in these cells (Asharani et al., 2010). The cytotoxicity of Pt nanomaterials has also been associated with genotoxic stress, DNA oxidation (Kutwin et al., 2017), and glutathione reduction (Pelka et al., 2009).

PPy NPs have been widely studied for application in tumor PTT due to their significant photostability and excellent photothermal

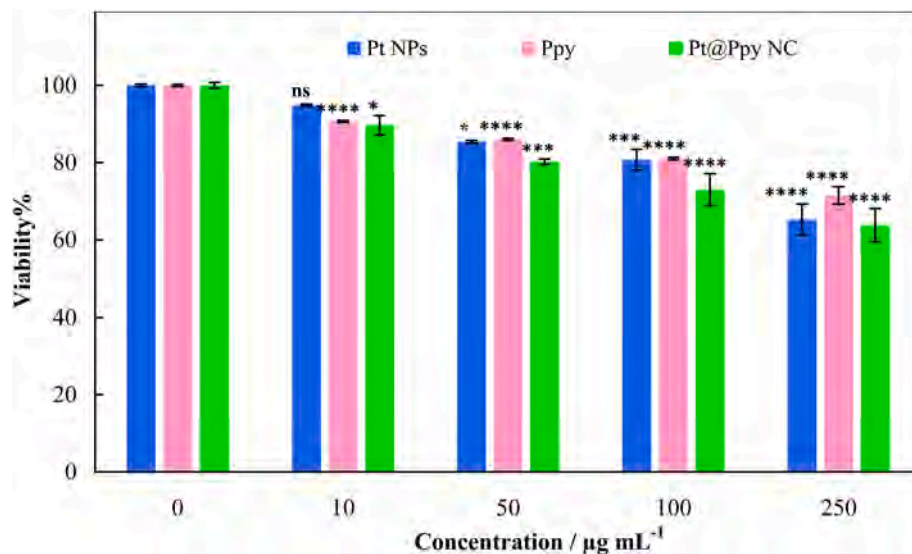


Fig. 4. In vitro viability percents of melanoma cells treated with different concentrations of Pt NPs, PPy NPs, and Pt@PPy NC. *Significant differences (P-value <0.05). **Very significant differences (P-value <0.01).

performance. A PPy-polyethyleneimine drug-free nanozyme induced cell decay via loss of mitochondrial transmembrane potential, hydropic mitochondrial swelling, antiapoptotic proteins reducing, and caspase-dependent apoptotic in B-cell lymphoma (Nguyen et al., 2023). The cytotoxic effect of PPy NPs on human T lymphocyte Jurkat cell line, primary mouse embryonic fibroblasts, and MH-22 A mouse hepatoma cell line was found to be dose-dependent. At low concentrations, PPy NPs demonstrated biocompatibility, whereas at higher concentrations they became cytotoxic to the cells (Vaitkuvienė et al., 2013). It seems that these results confirm the present findings on PPy NPs.

A drug-free nanozyme composed of polypyrrole and polyethyleneimine induced cell death, loss of potential of mitochondrial transmembrane, hydropic mitochondrial swelling, caspase-dependent apoptosis in B-cell lymphoma and downregulation of anti-apoptotic proteins (Nguyen et al., 2023).

Cytotoxicity of Pt NPs, PPy NPs, and Pt@PPy NC upon 808-nm light irradiation and US exposure (individually and synchronized) was evaluated after 24 h against the B16-F10 melanoma cell line using the MTT proliferation assay. Fig. 5 shows the viability of B16-F10 cells exposed to a concentration of $100 \mu\text{g mL}^{-1}$ of Pt NPs, PPy NPs, and Pt@PPy NC, and light irradiation and US exposure (individually and synchronized). Photosensitizing effect upon irradiation with 808-nm light as well as sonosensitizing effect upon exposure to US of Pt NPs, PPy NPs, and Pt@PPy NC is witnessed from the results. Also, the sonosensitizing effect of these nanostructures was more effective than photosensitizing one. On the other hand, synchronized light irradiation and US exposure induced higher cytotoxicity against B16-F10 cells for all the nanostructures (compared to individual radiation), and the highest cytotoxicity (viability of 27%) was attained upon synchronized radiations of Pt@PPy NC. Considering the viability values of the cells upon PTT, SDT, and PST, CIs greater than unity were obtained (Supplementary material S5), indicating domination of synergistic effects between the nanostructures and light, the nanostructures and US, and the nanostructures and synchronized light irradiation and US exposure, respectively. This confirms the most effectiveness of Pt@PPy NC for PST applications. It has been reported that porous Pt NPs coated with poly (diallyldimethylammonium chloride) upon exposure to high power 808-nm light (8.4 W cm^{-2} for 3 min) reduce the viability of human glioblastoma U-87 MG cells to 21% (Zhu et al., 2016). An Arg-Gly-Asp peptide-coupled polydopamine-modified mesoporous Pt NPs (at $100 \mu\text{g mL}^{-1}$) presented a PTT effect under 808-nm light of 1.5 W cm^{-2} with a reduced

viability of human ovarian adenocarcinoma SKOV-3 cells of 29%, disrupted the actin cytoskeleton, and inhibited tumor migration (Zou et al., 2022). Similarly, a viability of 26% for non-carcinogenic murine L929 cells was obtained upon treatment with Au@Pt-PEG ($100 \mu\text{g mL}^{-1}$) and near IR light irradiation (Zhou et al., 2021).

ROS production is the mainspring of cancerous cells killing in PTT upon heat stress, as well as in PDT and SDT upon irradiation of photo/sonosensitizers (Kayani et al., 2021; Perota et al., 2022). SDT with sonosensitizer also resulted in cytoskeletal and cell membrane damage (Rosenthal et al., 2004b). For affirming the sonosensitizing capacity and mechanism of action of Pt NPs, PPy NPs, and Pt@PPy NC during SDT, US-activated ROS generation was fluorescently detected by DCF in the absence of cellular intervention, and the obtained results are shown in Fig. 6. Enhanced fluorescence emissions were observed upon US exposure of Pt NPs, PPy NPs, and Pt@PPy NC, compared to the controls, suggesting a highly efficient US-activated ROS production.

Intracellular ROS production levels during PDT, SDT, and PST of B16-F10 cells were determined using H2DCF-DA. As shown in Fig. 7A, Pt NPs and PPy NPs alone produced almost the same ROS levels (31.9 and 35.4, respectively). On the other hand, considering the ratio of PPy and

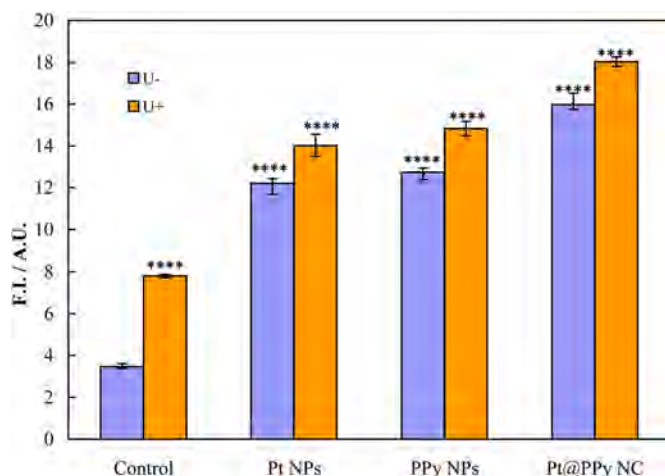


Fig. 6. Fluorescence emission of DCF in a cell-free system upon US radiation, and incubation with Pt NPs, PPy NPs, and Pt@PPy NC ($100 \mu\text{g mL}^{-1}$).

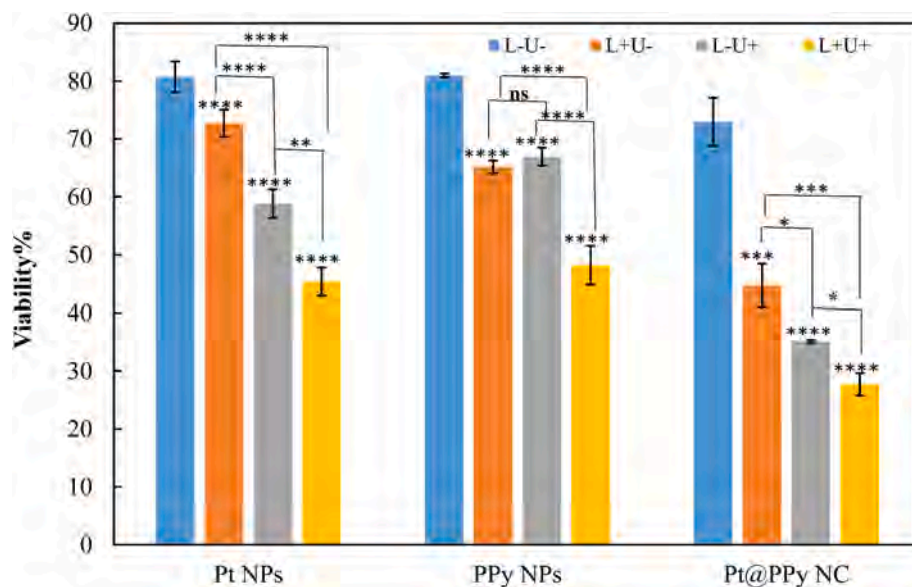


Fig. 5. In vitro viability percents of melanoma cells treated with Pt NPs, PPy NPs, and Pt@PPy NC ($100 \mu\text{g mL}^{-1}$) upon 808-nm light irradiation and US exposure (individually and synchronized).

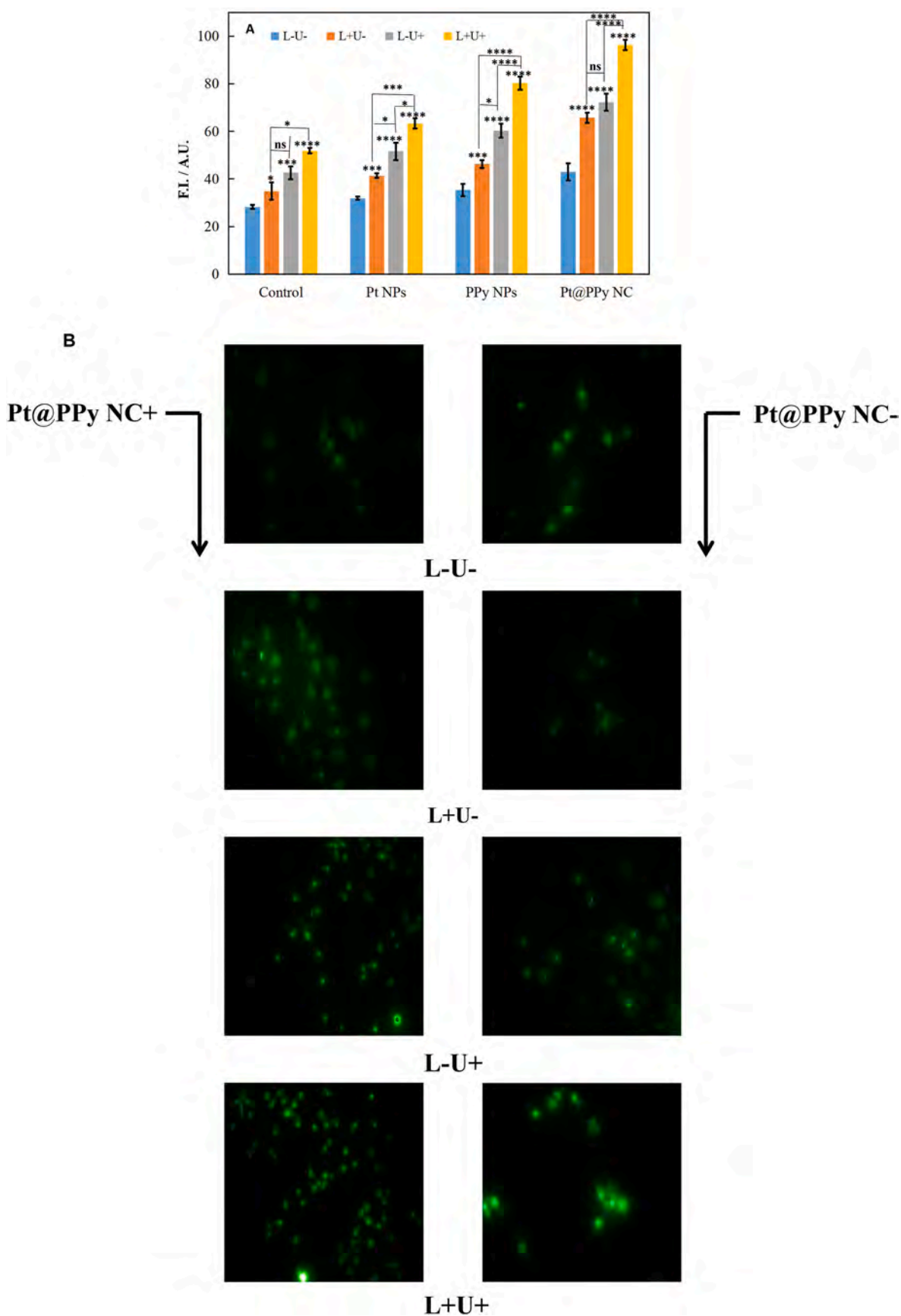


Fig. 7. Fluorescence intensity of intracellular DCF in different cell groups (A). *Significant differences (P-value <0.05). **High significant differences (P-value <0.001). ***Extremely significant differences (P-value <0.0001). Fluorescence images (10 × magnification) of DCF-stained B16-F10 cells showing intracellular ROS generation upon affections of laser light and US in absence or presence of 100 $\mu\text{g mL}^{-1}$ of Pt@PPy NC (B).

Pt in Pt@PPy NC (77.7 and 22.3%, respectively) and a simple calculation using the results of ROS generation levels by Pt NPs (31.9) and PPy NPs (35.4), it is revealed that the ROS level produced by Pt@PPy NC (43.1) was higher than summation of its components (34.6). Therefore, PPy and Pt in Pt@PPy NC represented a synergism effect to produce ROS. In addition, Pt NPs, PPy NPs, and Pt@PPy NC represented greater fluorescence emissions (compared to control) either when they were irradiated by 808-nm light or exposure to US, and the fluorescence emission of Pt@PPy NC was the highest (compared to the other nanostructures). Moreover, PST of the cells using the nanostructures was more effective than either PDT or SDT, and the highest level of ROS production was attained upon PST using Pt@PPy NC (P-value<0.001). Again, considering the ratio of PPy and Pt in Pt@PPy NC, and the results of ROS generation levels upon PDT, SDT, and PST using Pt NPs (41.4, 51.6, and 63.3, respectively), and using PPy NPs (46.3, 60.2, and 80.2, respectively), it is disclosed that the ROS level produced upon PDT, SDT, and PST using Pt@PPy NC (65.7, 72.3, and 96.2, respectively) were higher than those were expected (45.2, 58.3, and 76.4, respectively). It was further confirmed the synergism effects of Pt@PPy NC components to produce ROS under radiations too.

Fluorescence images of stained B16-F10 cells by DCF in the cell groups are also presented in Fig. 7B. In these images, the bright green spots indicate DCF accumulation in the cells due to ROS generation. The green spots were the lowest in the control cells, more green spots were observed upon irradiation with 808-nm light, US exposure or synchronized light irradiation and US exposure. Green spots elevated upon PDT, SDT, and PST, respectively, using Pt@PPy NC. ROS induces lipid peroxidation and potential depolarization of the mitochondrial membrane (Wan et al., 2016). DNA damage and lipid peroxidation contribute to cell decay via the apoptotic events (Hayyan et al., 2016).

Since the growth environment of tumor cells is acidity and hypoxia, the catalase-mimicking enzymatic activity of Pt nanostructures, which decomposes H_2O_2 into H_2O and O_2 , has been successfully exploited to alleviate tumor hypoxia (You et al., 2020a; Wei et al., 2018). In addition, Pt nanostructures have a peroxidase-like activity that enables them to decompose H_2O_2 into toxic OH radicals (Zhang et al., 2023). Fig. 8 shows UV-vis absorption spectra of Pt@PPy NC in the presence of H_2O_2 , immediately and after 180 min. The absorbance was decreased after incubation of Pt@PPy NC with H_2O_2 , and a H_2O_2 degradation ratio of 48.5% was obtained. These results highlighted the effective catalase activity of Pt@PPy NC, indicating that it can be potentially employed as an oxygen donor to enhance the efficacy of PDT and SDT in a hypoxic environment (You et al., 2020a). Furthermore, catalase has the potential to mitigate the adverse consequences associated with PDT, such as the occurrence of tissue damage resulting from the excessive presence of H_2O_2 (You et al., 2020b). Recently, oxygen generation in situ and reduction of oxygen consumption have been considered as adjuvant therapy to alleviate tumor hypoxia. In addition, Pt NPs, PPy NPs, and Pt@PPy NC as photo/sonosensitizers promoted ROS generation and oxidative stress within tumor cells that lead to apoptosis. Supplementary material S6 summarizes recent studies on melanoma cells that explore the anti-tumor responses elicited by various therapeutic modalities (chemotherapy, radiotherapy, immunotherapy, PDT, SDT, and combined therapies).

A potential clinical implementation of Pt@PPy NC as a sensitizer for melanoma cancer treatment would most likely follow a localized, image-guided delivery and activation strategy to maximize tumor selectivity and minimize exposure of healthy skin. Our results demonstrated cytocompatibility of Pt@PPy NC toward the normal cells. In practice, Pt@PPy NC could be administered intratumorally or by peritumoral injection, or can be delivered systemically after tumor-targeting modification to concentrate within the melanoma lesion. After accumulation, treatment would employ with 808-nm laser irradiation combined with US exposure. Selective cytotoxicity is expected because of ROS and heat generation in the tumor after local injection of Pt@PPy NC and its catalase-mimicking activity. Nonetheless, future studies need to validate

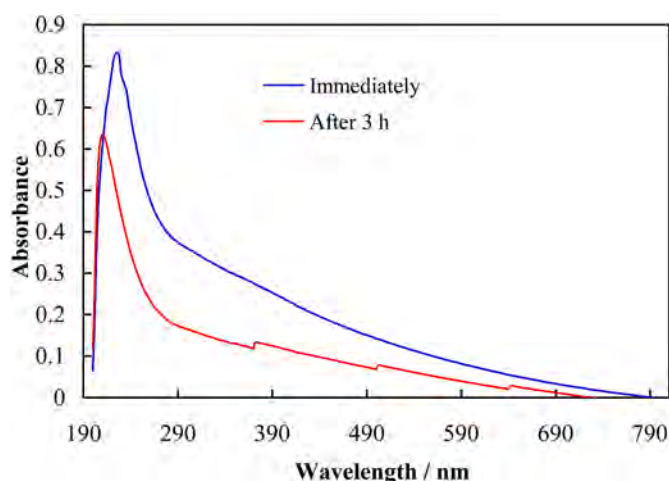


Fig. 8. UV-vis absorption spectra of Pt@PPy NC in the presence of H_2O_2 , immediately and after 180 min.

these selectivity mechanisms in melanoma animal models.

4. Conclusion

In summary, Pt@PPy NC was synthesized, and its absorption ability of 808-nm light was confirmed by UV-vis spectroscopy. It represented ROS production ability upon US exposure. The results of the MTT assay confirmed its PTT, SDT and PST capabilities to kill melanoma cancer cells with the highest efficacy attainable for PST. Pt@PPy NC also acted as a catalase-mimicking enzymatic activity that can generate molecular oxygen and alleviate tumor hypoxia. The oxygen generation ability provides suitable conditions for the best performance of ROS generation.

CRediT authorship contribution statement

H. Haghighi: Writing – original draft, Investigation, Formal analysis, Data curation. **P. Tamaddon:** Writing – review & editing, Investigation, Formal analysis. **P. Faghani-Eskandarkolaei:** Writing – review & editing, Investigation, Formal analysis. **H. Heli:** Writing – review & editing, Validation, Conceptualization. **M. Haghani:** Methodology, Investigation, Formal analysis. **N. Sattarahmady:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Ethical statement

This study does not contained animal or human study. All the procedures were approved by Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.MED.REC.1400.130).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank the Research Council of Shiraz University of Medical Sciences (23211) and the Iran National Science Foundation (4044184) for supporting this research. The authors would also like to thank Center for Development of Clinical Research of Nemazee Hospital and Dr. Nasrin Shokrpour for editorial assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtherbio.2026.104428>.

Data availability

Data will be made available on request.

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