


Highly Active Nano-Reactor for Responding Tumor Microenvironment and Antitumor Therapy

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Abstract

The tumor microenvironment is complex and changeable, so the design of a nano-delivery system for the tumor microenvironment has attracted wide attention. Based on this, we designed an intelligent nano-reactor for the characteristics of acidic pH and hypoxia in the tumor microenvironment. Firstly, the silver nano-balls were synthesized by the biological template method, which exhibited a good photothermal conversion efficiency and can realize the photothermal treatment of tumor sites. Subsequently, the hypoxic pro-drug tirapazamine (TPZ) and polydopamine (PDA) for chemotherapy were self-assembled. After PDA arrived at the tumor site (pH 5.5) from the normal physiological environment (pH 7.4), the hypoxic prodrug TPZ was released in pH response by PDA. Subsequently, TPZ selectively induced obvious cell damage under tumor hypoxia stimulation but had no toxic effect on normal cells under normal oxygen. In addition, the nano-converter was loaded with iRGD on the surface, which enhanced the targeted delivery of the nano-reactor to achieve a highly effective antitumor effect. The nano-reactor was capable of combining photothermal/chemotherapy therapy. Importantly, it can selectively kill tumor cells without damaging normal cells based on the characteristics of the tumor microenvironment, with high bio-safety and clinical transformation potential.

Keywords

tumor microenvironment, photothermal therapy, tirapazamine

Abbreviations

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Introduction

Cancer is still one of the most lethal diseases in the world. Although cancer research has made some progress, the incidence and mortality of cancer are increasing year by year.¹ In recent years, nanomedicines have developed rapidly in the field of antitumors, and made great breakthroughs in inhibiting tumor growth and metastasis. However, the heterogeneity and complexity of the tumor microenvironment (TME), such as acidic pH, hypoxia, and excess lactic acid, can limit the effects of nanomedicines. Scientists have carried out a lot of exploration in the complex tumor microenvironment, and the specific treatment caused by the tumor microenvironment has also attracted extensive attention.² Hypoxia is a characteristic of the tumor microenvironment and is mainly caused by vascular curvature and lymphatic deficiency. At the same time, hypoxia has gradually become a specific target of tumor therapy, such as hypoxic-specific drugs, hypoxic-cell radiosensitizers, and the use of hypoxic-induced target genes targeting

tumor tissues, and so on, have shown great application prospects.³ Tirapazamine (TPZ)⁴ is an effective hypoxic selective drug, which can release active ingredients by bioreductive metabolism with a strong cytotoxic effect in the hypoxic microenvironment of tumor and exert a therapeutic effect. In addition, TPZ, as a topoisomerase II agent, can induce DNA double-strand breaks, which can bind tumor cells to G2/M phase arrest and mitochondrial

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pathway-induced apoptosis. Hong *et al*⁵ showed that TPZ can induce growth inhibition of nasopharyngeal carcinoma cells under hypoxia, which is associated with significant cell cycle arrest and PARP division (a marker of apoptosis) in the S phase. The encouraging results indicated the potential of hypoxia-targeted therapy for anticancer.

Acid pH is one of the important characteristics of the tumor micro-environment. Normal tissues maintain a normal physiological environment due to their aerobic respiration (pH 7.4). However, due to the occurrence of the anaerobic glycolysis pathway, a large amount of lactic acid is produced in the tumor tissue, so the pH value is 5.5 to 6.6.⁶ In recent years, according to the acidic microenvironment inside the tumor, a series of acid pH-responsive nano-drug delivery and diagnosis and treatment integrated systems have been designed using pH-sensitive chemical bonds, polymers, and inorganic substances. Dopamine is a good pH-sensitive material, which can be synthesized to polydopamine under alkaline conditions and depolymerized under acidic conditions. Therefore, it can be used as a good carrier for pH-sensitive drug release. These nano-systems are relatively stable under normal conditions but can be activated by an acidic environment to release chemotherapeutic drugs at the tumor site.⁷ Li *et al* reported a pH-activated, near-infrared light-triggered photodynamic therapy system that was able to dissociate to facilitate the photoactivation of photosensitizers in a mildly acidic tumor microenvironment. The results of *in vitro* and *in vivo* experiments show that this treatment has a good antitumor effect and drug release rate.⁸

Chemotherapy alone could make tumor cells develop strong drug resistance, resulting in the survival of tumor stem cells, thereby inducing tumor recurrence and metastasis.⁹ Photothermal therapy kills all types of tumor cells without developing drug resistance, which also can make tumor cells more sensitive to chemotherapy drugs, inhibiting their DNA repair and induce lethal or sublethal cell damage.¹⁰ Therefore, the combination of photothermal therapy and chemotherapy is an effective and good antitumor strategy, and its therapeutic effect is far better than that of single chemotherapy.

Herein, aiming at the characteristics of acidic pH and hypoxia in the tumor microenvironment, we designed an intelligent nano-reactor AgNBs-PDA-TPZ-iRGD-lipid bilayer (AgPTIL). Firstly, AgNBs can realize photothermal therapy on tumor sites. When PDA reached the tumor site (pH5.5) from the normal physiological environment (pH7.4), PDA released TPZ in response to pH. Subsequently, TPZ as a hypoxic prodrug was used for chemotherapy. Subsequently, TPZ selectively induced significant cell damage under hypoxia stimulation of the tumor but had no toxic effect on normal cells under normal oxygen. In addition, the surface of the nano-reactor was loaded with iRGD, which enhanced the targeted delivery of the nano-reactor. The nano-reactor was capable of combining photothermal/chemotherapy therapy (Scheme 1).

Results and Discussion

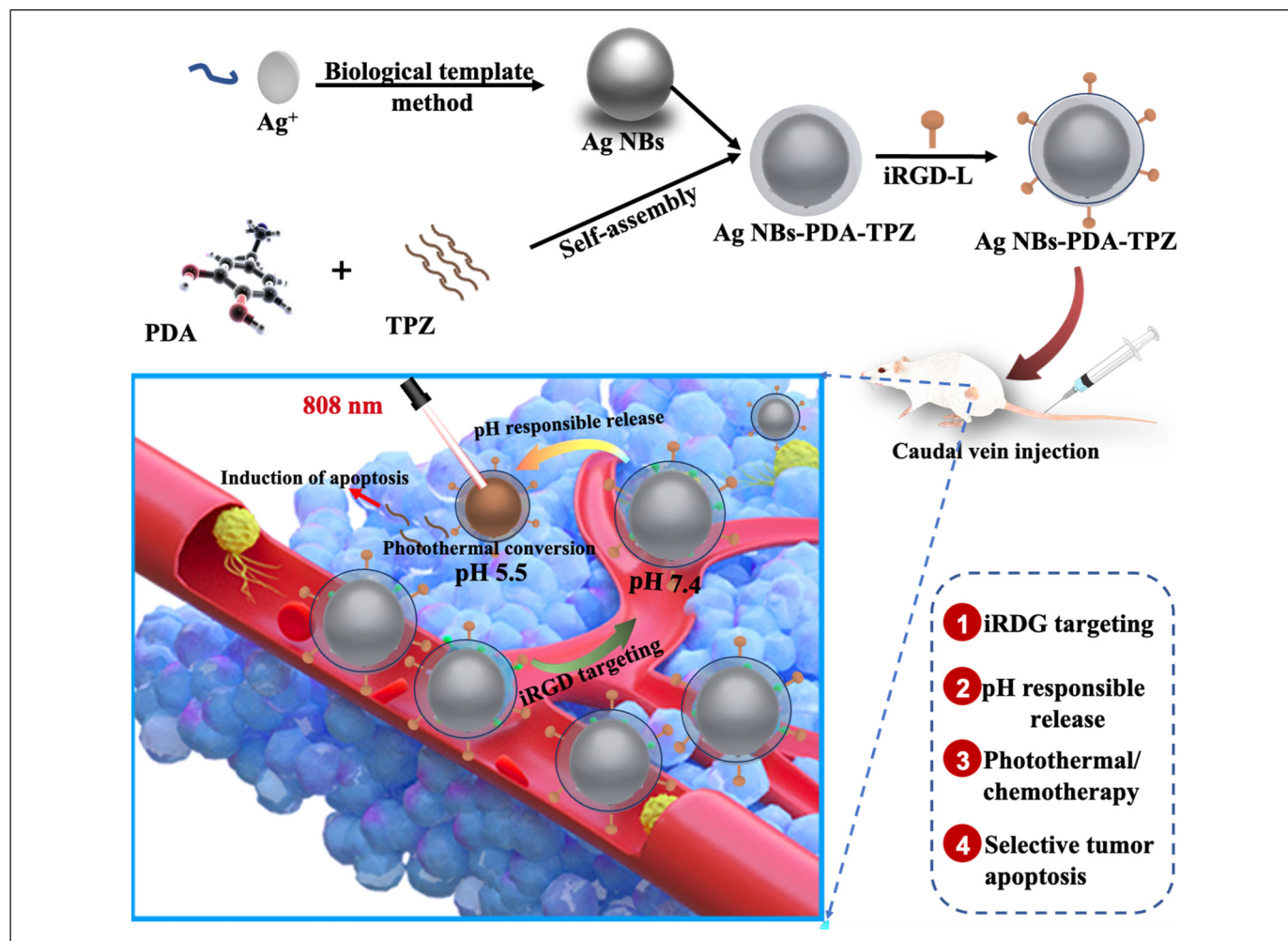
Characterizations of the AgPTIL

A transmission electron microscope (TEM) was used to characterize the morphology of different samples. As shown in

Figure 1A, Ag nanoballs with good dispersion were first synthesized, with a diameter of about 80 nm (Figure 1E and F). Then, the hypoxia prodrug TPZ was connected by electrostatic adsorption, and the dopamine was polymerized into PDA spherical shell under alkaline conditions to form AgNBs-PDA-TPZ (Figure 1B), which was about 150 nm (Figure 1F). As shown in Figure 1C, iRGD was connected inside the lipid bilayer to form a transparent microcapsule, and the iRGD-lipid bilayer (iRGD-L) was about 155 nm (Figure 1F). Finally, as shown in Figure 1D, iRGD-L was wrapped on the surface of AgNBs-PDA-TPZ to form AgNBs-PDA-TPZ@iRGD-lipid bilayer (AGPTIL), which was about 160 nm (Figure 1F). As shown in Figure 1G, AgNBs-PDA-TPZ was positively charged (28.8 ± 3.4 mV) and combined with negatively charged iRGD-L (-18.7 ± 1.8 mV) to form AGPTIL with a potential of about 10.6 ± 3.2 mV.

Photothermal Effect and Controlled Release *In Vitro*

The photothermal conversion ability of AgPTIL was tested. As shown in Figure 1H, AgNBs have good absorption at 800 nm, proving that it has good photothermal conversion potential, which is due to the surface plasmon resonance effect of the metal. In addition, PDA also has an absorption in the near-infrared region, which enhances the photothermal conversion capability of the final sample AgPTIL. As shown in Figure 2A, with the increase in irradiation time, the temperature of the AgPTIL increased gradually, and the temperature difference (ΔT) can reach 40 °C when the power was 2 W/cm² (808 nm). As shown in Figure 2B, the AgPTIL has obvious concentration dependence. With the increase in sample concentration, the photothermal temperature of AgPDIL increased faster. Subsequently, the photothermal stability of the AgPTIL was monitored at 808 nm (2 W/cm²), and the results showed that the maximum temperature of the AgPTIL did not change significantly in the 5 cycles (Figure 2C). Among them, AgNBs had good photothermal stability, which might be because the structure of Ag wrapped by PDA was not easy to change. Then the photothermal conversion ability of the AgPTIL was monitored by a near-infrared camera. As shown in Figure 2E, the solution in the EP tube gradually heated up as the irradiation time increased, showing a bright white color at a temperature above 60 °C after 4 min. In addition, the photothermal conversion efficiency of AgPTIL was calculated as 26.2%. Since TPZ would be released by PDA under the stimulation of NIR and pH, the release efficiency of TPZ was monitored. As shown in Figure 2D, when pH was 7 and temperature was 37 °C, the release efficiency of TPZ was less than 10% within 8 h, which proved that TPZ has good stability and avoided toxic and side effects caused by leakage in the body. When pH changed to 5.5 and approached the temperature of the acidic microenvironment of the tumor, the release efficiency of TPZ reached 90% in 24 h, so responsive release of the tumor microenvironment could be achieved. On this basis, when the temperature was raised to 45 °C, the drug release efficiency of TPZ was close to 100% within 8 h.



Scheme 1. Preparation process of AgNBs-PDA-TPZ-iRGD-lipid bilayer (AgPTIL) and in vivo antitumor treatment.

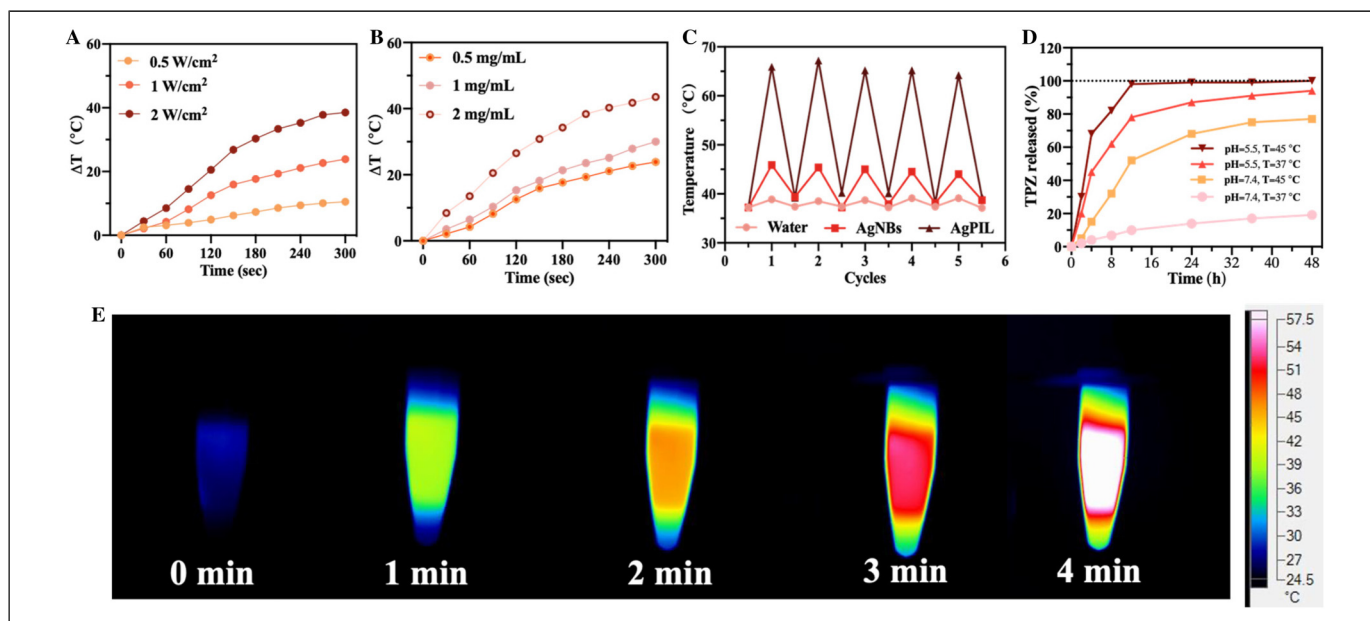
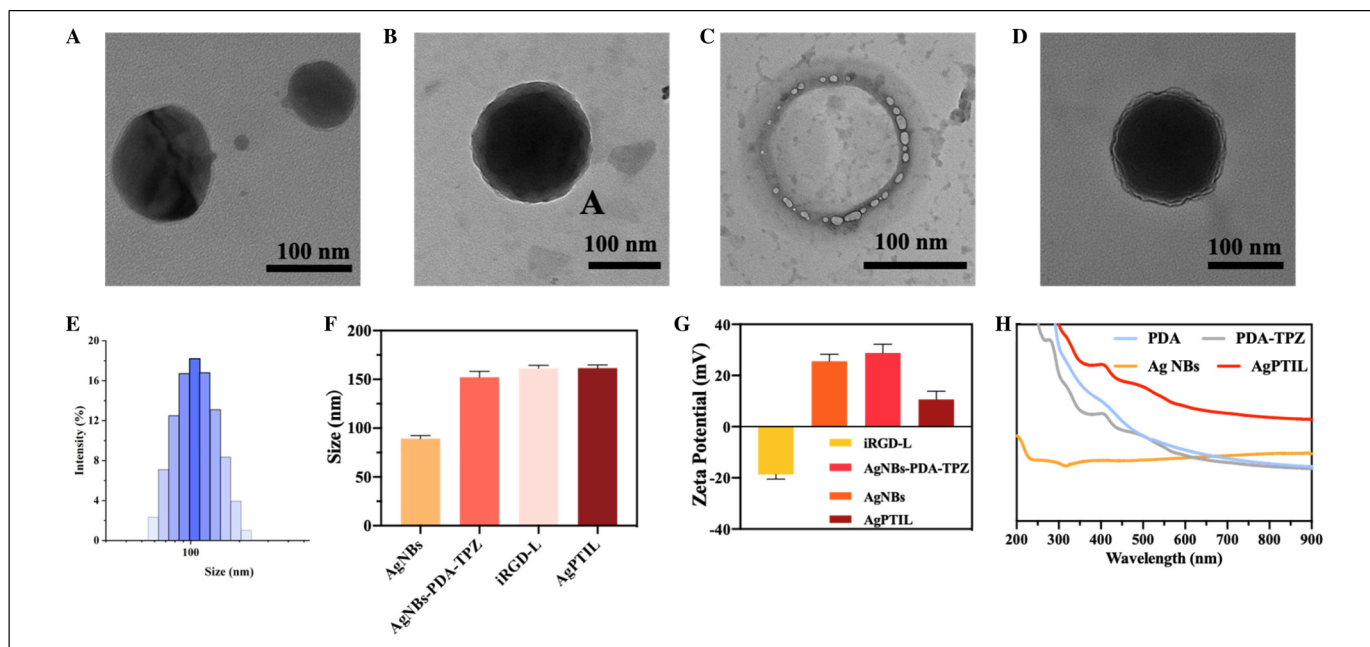
Enhanced Uptake and Antitumor Effect In Vitro

The enhanced uptake of HeLa cells was detected. The TPZ was replaced by DOX due to the red fluorescent of DOX, which was good for the positioning of samples. As shown in Figure 3A, the red fluorescent of DOX from AgNBs-PDA-DOX + NIR was less than that of AgPDIL + NIR, which suggested that the iRGD could promote the uptake of HeLa cells due to the targeting ability of tumor. In addition, the red fluorescent from AgPDIL was less than that of AgPDIL + NIR, which suggested that the NIR could improve the uptake of tumor cells. In order to test the antitumor activity of the AgPTIL, HeLa cells were used to detect cell survival rates after incubation with each sample by standard MTT assay. The results were shown in Figure 3B. Under laser irradiation, the cell survival rate of AgNBs was higher than that without laser, indicating that AgNBs had an excellent photothermal effect and tumor-killing effect. As an active prodrug under hypoxia, TPZ can cause obvious cell damage under hypoxia conditions, and induce relatively weak toxicity. Therefore, the antitumor effects of AgPTIL under the hypoxia and normoxia conditions were detected. As shown in

Figure 3C, the cell survival rates under the hypoxia condition were lower than those in normoxia condition, and the survival rate was decreased with the increase in concentration of TPZ. The results suggested that the TPZ could selectively damage tumor cells under the hypoxia environment at tumor sites. Then, the antitumor effects of different treatment groups were compared (Figure 3D). The cell survival rate of the AgNBs + NIR group was about 72.12%, while of the AgNBs-PDA + NIR therapy group was enhanced (54.31%). And the cell survival rate of the combination group was about 13.21% (AgPTIL + NIR), which was significantly higher than the chemotherapy treatment group. In addition, compared with the AgNBs-PDA-TPZ + NIR group, the cell survival rate of the AgPTIL + NIR group decreased to 13.21%, indicating that iRGD provided a targeting capability, and the increased intracellular uptake would enhance its tumor-killing effect.

Antitumor Effect In Vivo

As shown in Figure 4A, the expression level of HIF-1 α in normal tissues was about 20 pg/mL, while in tumor tissues it was about



70 pg/mL. Then, the mRNA expression of HIF-1 α was monitored in tumor cells. As shown in Figure 4B, the mRNA level of HIF-1 α in normal tissues was lower than that of tumor tissue, which suggesting the tumor tissue exhibited hypoxia to active TPZ. In addition, the light diffraction images also showed that

the normal tissue appeared bright red, while the tumor appeared blue, indicating that oxygen levels were much higher in the normal tissue than in the tumor (Figure 4C and D).

Subsequently, the antitumor effects of different samples were tested, as shown in Figure 5A and B. The relative tumor

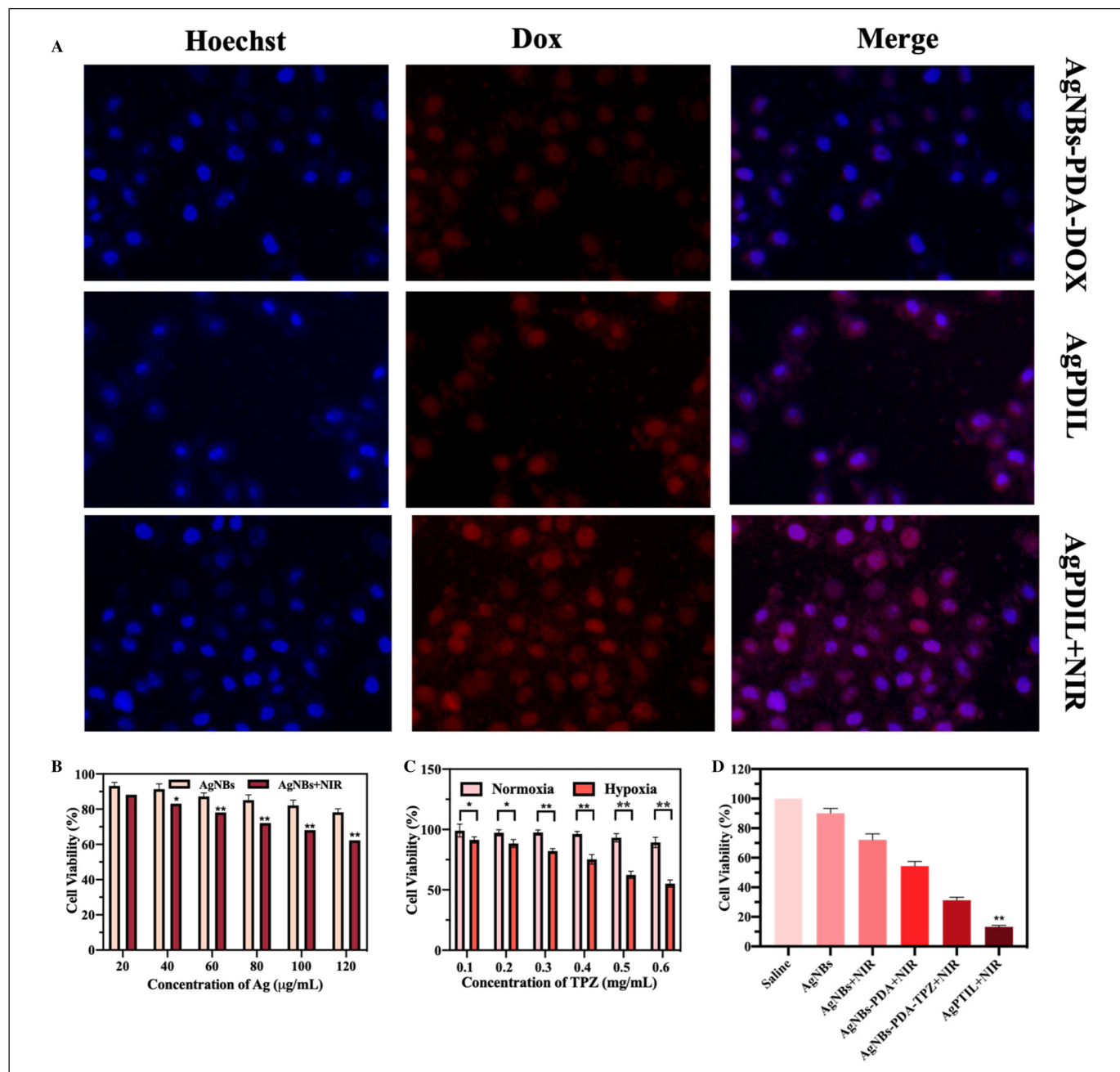


Figure 3. (A) Fluorescent microscopy images of uptake of HeLa cells cultured with different samples. (B) Cell viability of HeLa cells treated with AgNBs with or without NIR laser irradiation (808 nm , 1.5 W/cm^{-2}). (C) Cell viability of HeLa cells treated with different concentrations of AgPTIL under the normoxia, or hypoxia conditions with NIR laser irradiation (808 nm , 1.5 W/cm^{-2}). (D) Cell viability of HeLa cells treated with different treatments (808 nm , 1.5 W/cm^{-2}). * $P < .05$, ** $P < .01$.

volume and tumor weight of mice in the normal saline group were about 17.4 and 2.5 g, respectively, which were significantly higher than those in the simple photothermal therapy group (AgNBs + NIR), the combined therapy group (AgNBs-PDA-TPZ + NIR) and the combined therapy group with a targeted ability (AgPTIL + NIR). As shown in Figure 5D, the representative photographs of tumor-bearing mice were shown, showing that the final group had a favorable antitumor effect, with smaller tumor volumes than the other

groups, and almost no tumor presence compared to the saline group. In addition, the antitumor effect of the combined treatment group (AgPTIL + NIR) was much better than that of the chemotherapy group alone (TPZ-L) or the phototherapy group alone (AgNBs + NIR). Produced mainly by activated immune cells, TNF- α is a factor that kills tumor cells in vivo or inhibits their proliferation. Therefore, the expression of TNF- α was monitored to judge the degree of tumor cell injury. As shown in Figure 5C, the expression level of the

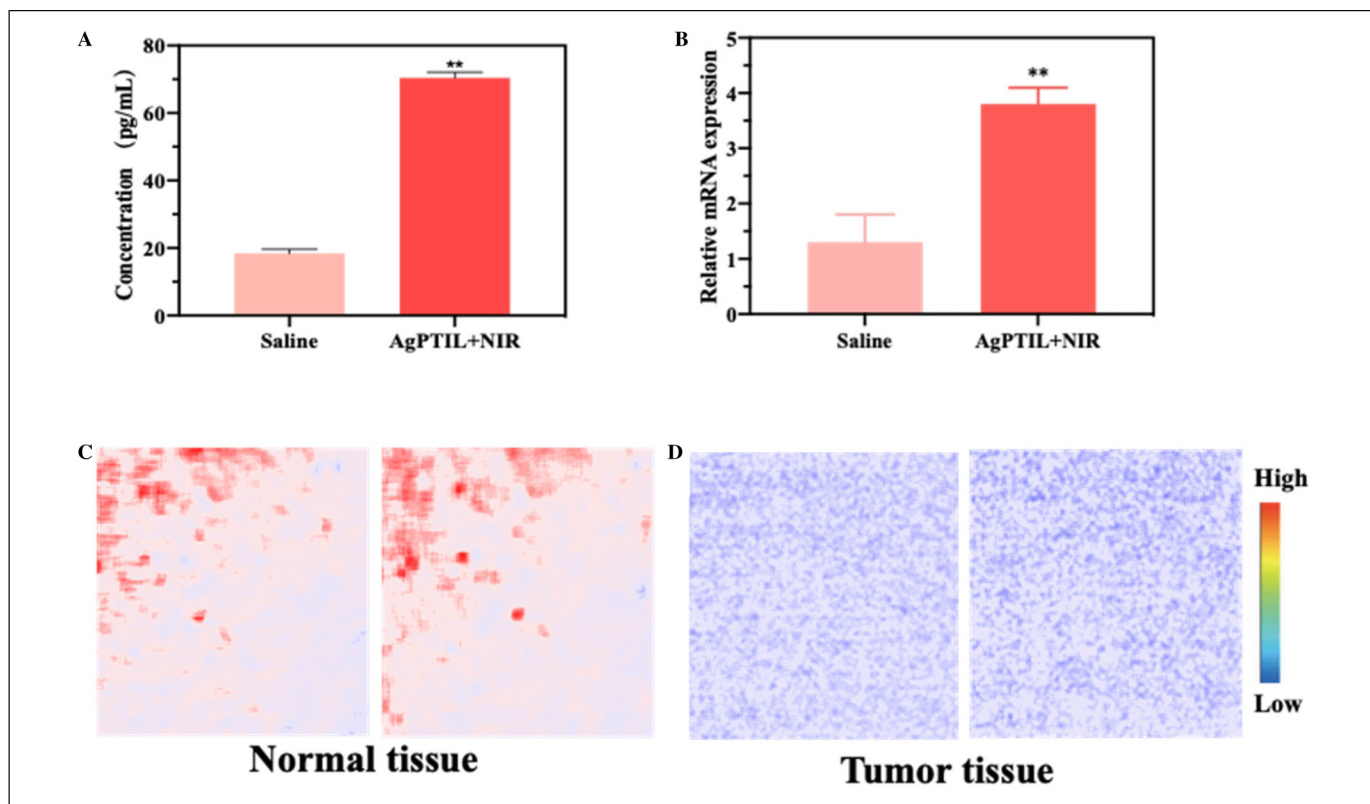


Figure 4. (A) Expression levels of HIF-1 α in mice with saline and AgPTIL + NIR. (B) The mRNA levels of HIF-1 α in mice treated with Saline and AgPTIL + NIR. Oxygen content in normal tissue (C) and tumor tissue (D). * $P < .05$, ** $P < .01$. Abbreviations: HIF-1 α , hypoxia-inducible factor-1 α ; AgPTIL, AgNBs-PDA-TPZ-iRGD-lipid bilayer; NIR, near-infrared.

AgPTIL + NIR group was about 11.1 pg/mL, which was higher than that of the saline group, which proved that AgPTIL + NIR had a good tumor-killing effect.

Potential Toxic Effects

As shown in Figure 6A, the bodyweight of mice in each treatment group did not change significantly during the treatment process, suggesting that the AgPTIL had little systemic toxicity on mice. In addition, serum analysis was performed using blood urea nitrogen (BUN),⁹ alanine aminotransferase (ALT), and aspartic aminotransferase (AST) as indexes (Figure 6B to D). Serum aspartate aminotransferase (AST) and ALT are generally used to analyze hepatotoxicity⁰, and BUN is an indicator of renal function.^{11,12} The results showed that the serum biochemical indexes of the AgPTIL + NIR group were basically the same as those of the normal saline group after 14 days, further confirming the good biological safety of AgPTIL.

Discussion

First of all, we prepared AgPTIL, and proved its successful preparation through various characterization, such as TEM, Zeta potential, and so on. Ag has good photothermal conversion ability because of its plasma resonance effect. In addition,

because of its good absorption in the near-infrared (NIR) light, PDA also has a good photothermal conversion ability. Ag NBs and PDA have good absorption in the near-infrared region, which proves that they have good photothermal conversion ability (the photothermal conversion efficiency was 26.2%). AgPTIL has obvious concentration dependence and good photothermal stability, which may be because the structure of Ag wrapped by PDA was not easy to change. In addition, because PDA could release TPZ under the stimulation of NIR and pH. When the pH value was 5.5, the release efficiency of TPZ can reach 90% within 24 h, which can realize the responsive release of the tumor microenvironment. On this basis, when the temperature was increased to 45 °C, the release efficiency of TPZ was close to 100% within 8 h, proving that NIR and pH can be used as a good switch to achieve reactive drug release, which can not only achieve accurate treatment of tumors but also have good biosafety.

The iRGD is a cyclic peptide composed of 9 amino acids, which can promote the tissue penetration of drugs, and has the effect of targeting tumors and tumor penetration. The uptake of AgPDIL was the strongest under the NIR, which suggested that iRGD and NIR could promote the uptake of tumors. On this basis, the AgPDIL also showed a good antitumor effect in vitro.

Hypoxia is a feature of the tumor microenvironment, so we monitored the degree of hypoxia at the tumor site and the therapeutic effect of TPZ. The hypoxia-inducible factor-1 α

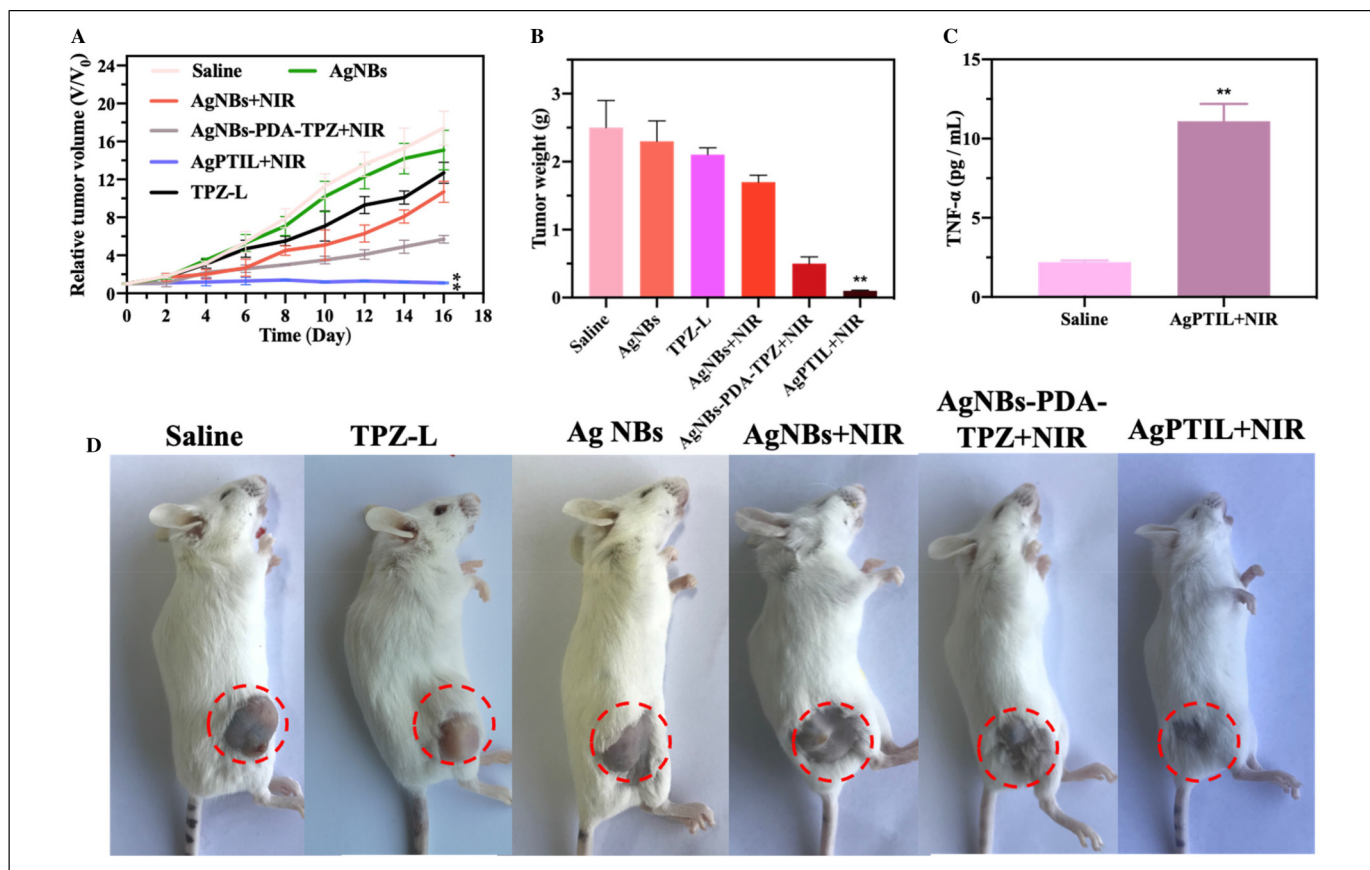


Figure 5. (A) Tumor growth profiles in various groups of the mice after 14 days of different treatments. (B) Tumor weight of various groups of mice after 14 days of different treatments. (C) The expression level of TNF- α in tumors with different treatments. (D) Representative photographs of tumor-bearing mice after various treatments for 14 days. $n = 6$, error bars represent \pm SD. $n = 6$, error bars represent \pm SD. * $P < .05$, ** $P < .01$.

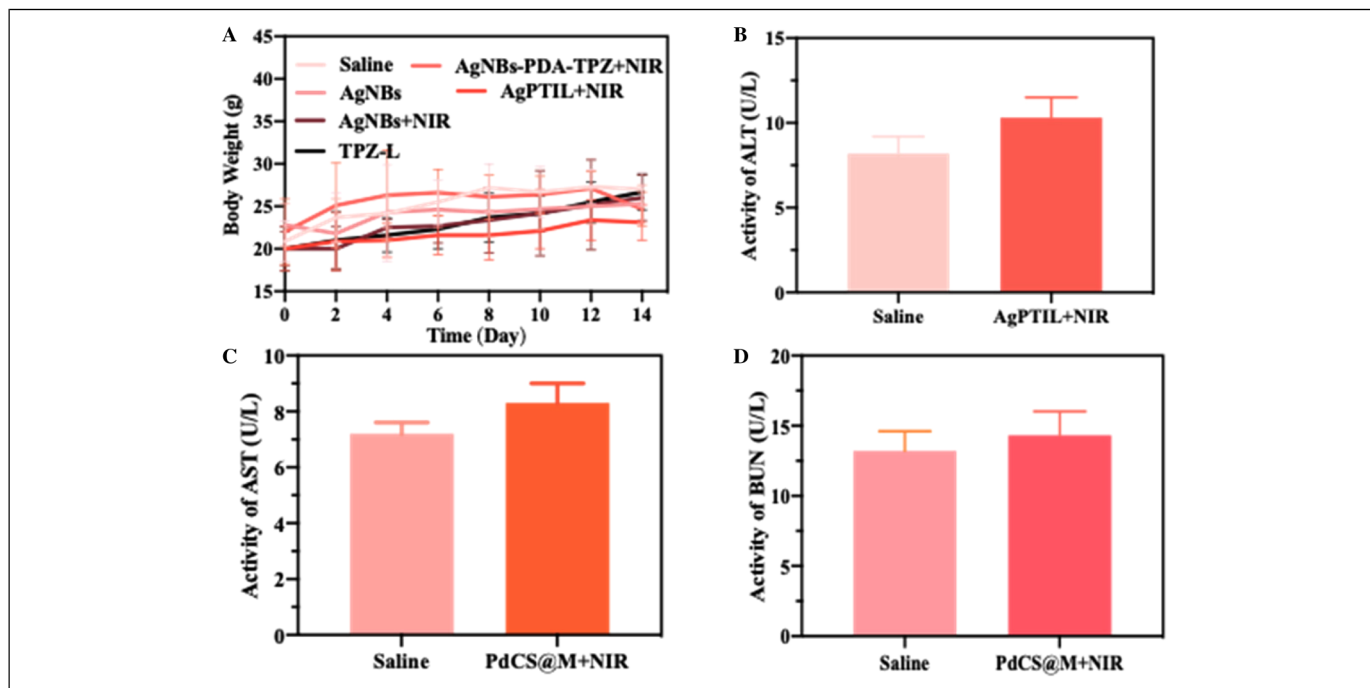


Figure 6. (A) Body weights of mice treated with different samples. Serum biochemistry dates of ALT (B), AST (C), and BUN (D) of the mice treated with saline and AgPTIL. $n = 6$, error bars represent \pm SD. * $P < .05$, ** $P < .01$. Abbreviations: ALT, alanine aminotransferase; AST, aspartic aminotransferase; BUN, blood urea nitrogen; AgPTIL, AgNBs-PDA-TPZ-iRGD-lipid bilayer.

(HIF-1 α) was quickly degraded by the intracellular oxygen-dependent ubiquitin-protease degradation pathway in normal oxygenated cells.¹³ HIF-1 α expression was stable only under hypoxia conditions, so we monitored the expression of HIF-1 α to reflect hypoxia in the tumor sites. Tumor tissue showed activation of TPZ by hypoxia, and the oxygen content of normal tissue was much higher than that of the tumor. Subsequently, the antitumor effects of different samples were tested. The results showed that the tumor growth in the AgPTIL + NIR group was completely inhibited, and the final tumor weight was only 0.1 g. The results indicated that the combined treatment group has a dual antitumor effect, which was photothermal therapy and chemotherapy respectively. In addition, hypoxia, acidic pH, and NIR at the tumor site can effectively control the release and activation of TPZ, thus achieving enhanced antitumor effects. The representative photographs of tumor-bearing mice showed that the final group had a favorable antitumor effect.

Biosafety is crucial for the application of nanomaterials in biomedicine. In the study, the biosafety was evaluated by observing the changes in body weight of mice in each group during the course of administration. The bodyweight of mice in each treatment group did not change significantly during the treatment process, suggesting that the AgPTIL had little systemic toxicity to mice.

Conclusion

Aiming at the characteristics of hypoxia and acidic pH in the tumor microenvironment, this study designed a double-responsive release nano-reactor and realized the switch of antitumor therapy through the stimulation of near-infrared light. The results showed that the loading of iRGD enhanced the targeting effect of the nano-reactor, the combination of photothermal therapy and chemotherapy achieved a good antitumor effect, the nano-reactor had good biosecurity, and the serum indicators were no significant difference compared with the normal saline group. It can selectively kill tumor cells without damaging normal cells according to the characteristics of the tumor microenvironment, which has high biosafety and clinical transformation potential. Therefore, the design of our nano-reactor provides a new idea for nanomedicine targeting the tumor microenvironment. In addition, serum analysis further confirmed the good biological safety of AgPTIL. These results provide the possibility for its application in the biomedical field.

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Ethical Statement

In “Highly active nano-reactor for responding tumor microenvironment and antitumor therapy” (TCRT-21-1393), all animal procedures were performed in accordance with the statute of the laboratory animal ethics committee of Northern Theatre General Hospital to ensure the low suffering for the animals, which were approved with

the statute of Experimental Animal Ethics Committee of Northern Theatre General Hospital China in 2020-12-25. The ethical review board approval number is 2020005.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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