Specific Photothermal Ablation Therapy of Endometriosis by Targeting Delivery of Gold Nanospheres

Xiaomeng Guo, Wei Li, Jialin Zhou, Wanqing Hou, Xue Wen, Hanbo Zhang, Fenfen Kong, Lihua Luo, Qingpo Li, Yongzhong Du, and Jian You*

1. Introduction

Endometriosis is difficult to treat since the side effects of the current therapeutic method and the high recurrence rate; thus, newer and safer therapeutic approaches are urgently needed. This work investigates the enhanced permeability and retention effect of CdTe quantum dots (QDs) and hollow gold nanospheres (HAuNS) in endometriosis to increase the delivery of HAuNS into lesion cells. The surface of HAuNS is successfully conjugated with a TNYL peptide that has specific affinity for the EphB4 receptor, which is a member of the Eph family of receptor tyrosine kinases. It is found that the EphB4 receptor is overexpressed in endometriosis lesions. The data indicate that both QDs and HAuNS can efficiently accumulate in endometriotic lesions through permeable vessels and the TNYL-conjugated HAuNS (TNYL-HAuNS) accumulate more via the interaction with EphB4. The specific photothermal ablation therapy based on TNYL-HAuNS significantly inhibits the growth of the endometriotic volume and induces the atrophy and degeneration of ectopic endometrium with no detectable toxicity to the normal organs. The level of TNF-α and estradiol also significantly decreases in the endometriotic lesions, indicating that the treatment enables a recovery from hormonal imbalance and inflammatory injury. This work can be a valuable reference for future endometriosis therapy.
vasomotor symptoms, and mood instability. New strategies are required to cause regression of the disease and symptoms without adverse hypoestrogenic effects.

Currently, nanoparticle-based platforms have emerged as suitable vehicles for cancer and inflammation treatments. Similar to solid tumors, endometriosis shows a high synovial proliferation and leaking of the associated blood capillaries, which motivate attempts to treat it using nanotechnology. Recently, mesoporous silica nanoparticles loaded with an immune modulatory drug were used on women with endometriosis by depressing the stimulatory effect of glucosaminyl muramyldipeptide (GMDP) on the membrane expression of scavenger receptors SR-AI and SR-B. Polymeric micelles compressing Pigment epithelium derived factor (PEDF) plasmid were used to treat endometriosis by suppressing angiogenesis. In another study, curcumin and letrozole were encapsulated in poly(lactic-co-glycolic acid) (PLGA) nanoparticles, and their efficacy in treating the disease was tested. However, these studies had limited therapeutic efficiency against endometriosis because of the lack of specific treatment and the potential systemic toxicity. A safer and more effective strategy to treat endometriosis based on nanotechnology must be further developed.

Photothermal ablation (PTA) therapy uses an optically absorbing agent (such as various gold nanostructures, carbon nanomaterials, CuS nanoparticles, etc.) to generate heat under near-infrared (NIR) laser irradiation, which results in a local high temperature that kills cells in diseased lesions while doing little harm to the native tissue during the treatment. Previously, we synthesized a class of gold nanoparticles at a large scale: hollow gold nanospheres (HAuNS), which have plasmon absorption in the NIR region and display a strong photothermal conduction property. The unique combination of a small size (30–50 nm in diameter) and a strong and tunable (520–950 nm) absorption band enables HAuNS to be promising photosensitizers for the photothermal therapy of cancer or other diseases. We hypothesized that HAuNS-based PTA could also induce efficient therapy against endometriosis with the mediation of an NIR laser. We suggest that the high accumulation and retention of HAuNS in an endometriosis site should be the essential component of NIR-mediated PTA treatment. In this work, two types of nanoparticles with different sizes (water-soluble CdTe QDs and HAuNS) were used to investigate the enhanced permeability and retention (EPR) effect on endometriosis lesions. Based on the high accumulation of the nanoparticles, HAuNS were used to treat endometriosis because of their high efficiency in photothermal conversion under NIR laser irradiation. To improve the specific PTA effect, EphB4 receptor expression in endometriosis lesions was investigated as a marker of high neovascularization. We introduced a peptide (TNYL) to the surface of HAuNS to enhance more nanoparticle accumulation in the endometriosis site because the peptide has a high binding affinity to the EphB4 receptor, as shown in our previous study. Our data indicate that relative to the conventional treatment for endometriosis, the HAuNS application exhibits superior therapeutic efficacy under NIR laser mediation. Our work includes a proof of concept study to develop a favorable strategy for endometriosis treatment.

2. Results and Discussion

2.1. Synthesis of CdTe QDs, HAuNS, and TNYL-Conjugated HAuNS (TNYL-HAuNS)

Figure 1A shows representative transmission electron microscopy (TEM) images of CdTe QDs with an ultrasmall size (8.42 ± 1.4 nm) and favorable monodispersity. The corresponding average hydrodynamic diameter in water was 9.42 ± 1.4 nm (Figure S1, Supporting Information), as measured using a dynamic light scattering (DLS) instrument. QDs with different emission wavelength in the NIR range (λmax = 530–730 nm) were readily obtained by adjusting the reaction time (0.5–14 h) (Figure S2 and Table S1, Supporting Information). Increasing the reaction time detectably enhanced the photothermal conduction property. The unique combination of a small size (30–50 nm in diameter) and a strong and tunable (520–950 nm) absorption band enables HAuNS to be promising photosensitizers for the photothermal therapy of cancer or other diseases. We hypothesized that HAuNS-based PTA could also induce efficient therapy against endometriosis with the mediation of an NIR laser. We suggest that the high accumulation and retention of HAuNS in an endometriosis site should be the essential component of NIR-mediated PTA treatment. In this work, two types of nanoparticles with different sizes (water-soluble CdTe QDs and HAuNS) were used to investigate the enhanced permeability and retention (EPR) effect on endometriosis lesions. Based on the high accumulation of the nanoparticles, HAuNS were used to treat endometriosis because of their high efficiency in photothermal conversion under NIR laser irradiation. To improve the specific PTA effect, EphB4 receptor expression in endometriosis lesions was investigated as a marker of high neovascularization. We introduced a peptide (TNYL) to the surface of HAuNS to enhance more nanoparticle accumulation in the endometriosis site because the peptide has a high binding affinity to the EphB4 receptor, as shown in our previous study. Our data indicate that relative to the conventional treatment for endometriosis, the HAuNS application exhibits superior therapeutic efficacy under NIR laser mediation. Our work includes a proof of concept study to develop a favorable strategy for endometriosis treatment.

2.2. Photothermal Conversion Effect of HAuNS and TNYL-HAuNS In Vitro

Continuous exposure of the aqueous suspensions of HAuNS and TNYL-HAuNS to NIR light rapidly increased their temperatures because of the plasma resonance absorption of the nanoparticles in the NIR region (Figure 1D). For example, at 1.5 W cm−2 and an equivalent Au concentration of 15 μg mL−1, the temperatures of the suspensions (HAuNS and TNYL-HAuNS) increased by 59.7 and 63.2 °C after 10 min of exposure, respectively. In comparison, no significant temperature change was observed when phosphate buffered saline (PBS) was exposed to laser light. TNYL-HAuNS had a similar temperature increase to that of HAuNS under identical irradiation conditions. All of these results are in accordance with our previous study and demonstrated the excellent photothermal conversion efficiency in vitro, which lays the foundation for photothermal therapy in vivo.
2.3. Cytotoxicity Assay

A significant dose-dependent decrease in cellular viability was observed, with lower concentrations of QDs leading to lower cytotoxicity.\[15\] As shown in Figure 1E, the cell line maintained only 50% cell viability after 24 h of treatment with QDs at a concentration of 2.8 \( \mu \)g mL\(^{-1}\). Relative to QDs, both HAuNS and TNYL-HAuNS had a significantly lower cytotoxicity and consequently higher biocompatibility.\[16\] The cells maintained 80% cell viability after 24 h of treatment at a Au concentration of 100 \( \mu \)g mL\(^{-1}\) (Figure 1F).

2.4. Characterization of Endometriotic Lesion in Model Mice

The endometriosis model in mice was considered successful because the endometrial explants developed into ovoid, large, fluid-filled, well-vascularized, and cystic lesions (Figure 2A–C).\[2a\] Endometriotic lesions with a volume growth of up to \( \approx 180 \) mm\(^3\) were observed on day 10. The morphology of the endometriotic lesion surface was examined using a scanning electron microscope (SEM) (Figure 2D,E), which shows that the lesions were in a compact arrangement with a tight structure. Furthermore, many new microvessels appeared in the endometrial stroma, as indicated by anti-CD31-positive staining (Figure 2F), and excess endometrial glands were observed from hematoxylin and eosin (H&E) staining (Figure 2G).

To further investigate the characteristic of the endometriotic lesion, the normal mouse uterus, congestive uterus, and endometriotic lesion were collected and analyzed for the levels of TNF-\(\alpha\) and estradiol using ELISA (Figure 2H,I). TNF-\(\alpha\) remained at a low level (\( \approx 1 \) pg mg\(^{-1}\) protein) in the normal mouse uterus and congestive uterus, whereas the TNF-\(\alpha\) level in the endometriotic lesions was six times of that in the normal uterus tissue of the mice (Figure 2H). The estradiol concentration of the normal uterus was 1.8 ng mg\(^{-1}\) protein, but it increased to 6.9 ng mg\(^{-1}\) protein when the uterus was in the congestion state. The estradiol concentration in the endometriotic lesion was slightly higher than that in the noncongestive uterus (Figure 2I). The expressions of the representative inflammatory factor and estrogen in the lesions were higher than those of the normal uterus tissue, which further demonstrates that the endometriosis model in mice was successfully established.

2.5. Expression of the EphB4 Receptor in the Endometriotic Lesion

The immunohistochemistry experiment was conducted to identify the expression of the EphB4 receptor in the endometriotic lesions, and the green fluorescent signal indicates the location of the receptor (Figure 2J). For normal uterine tissue, weak fluorescence was observed at either the edge
or the center of the uterus, which indicates a low expression of the EphB4 receptor. When the uterus was in a state of congestion, the expression of the EphB4 receptor was significantly increased. In the endometriotic lesions, a stronger green fluorescent signal was observed at the edge and center of the lesion, which suggests a high expression of the EphB4 receptor. The results of the Western blotting analysis also indicate a high expression of EphB4 protein: the percentage of EphB4 protein was 82.4% as calculated by the ImageJ software, whereas the percentages for the normal and congestive uterus were 0% and 52.3%, respectively (Figure 2K,L). The results are consistent with the immunohistochemistry experiment.

Thus, we modified HAuNS with the EphB4-receptor-targeted TNYL peptide to specifically treat endometriosis (Figure 2M). SH-PEG-TNTL peptide can be easily conjugated to the surface of HAuNS through a reaction between the thiol group and the Au. After an intravenous injection of TNYL-HAuNS, the nanoparticles were target-delivered into the lesions via the mediation of EphB4, and the lesions were expected to be destroyed by the HAuNS-generated heat under the 808 nm laser.

2.6. Biodistribution of CdTe QDs, HAuNS, and TNYL-HAuNS in Endometriotic Lesions

To identify the EPR effect in the endometriotic lesions, near-infrared-emitting CdTe QDs were first intravenously injected into the mice with bilateral lesions (Figure 3A). The biodistribution and selective accumulation in the endometriotic lesions were analyzed using an in vivo imaging system at predetermined time points (Figure 3B; Figure S5A, Supporting Information). Unambiguous fluorescence signals were observed at 12 h postinjection in the lesions on both sides and remained for at least 144 h, and the fluorescence intensity of QDs was decreased from 96 h postinjection (Figure S5A, Supporting Information). The fluorescence images of the major organs and lesions were obtained at 72 h after the injection (Figure 3C), and the fluorescence intensity in various tissues was quantified (Figure 3D). The results indicate that the QDs mainly accumulated in the liver and lesions. The amounts of QDs in the lesions (represented as the fluorescence intensity) were 16.89 ± 2.4 (left-side lesion) and 18.25 ± 2.3 (right-side lesion) ID% g⁻¹. The heart, liver, spleen, and lesions were also harvested and cut into slices to examine...
the distribution of QDs (Figure 3E). Their fluorescence intensity was quantified using ImageJ software (Figure S6, Supporting Information). The red fluorescence signals of QDs in the liver and lesion were significantly higher than those in the heart and spleen and persisted for least 72 h. Inductively coupled plasma mass spectrometry (ICP-MS) was used to further determine the amount of Cd atoms in the lesions, indicating that 4.95 ± 0.27 (left-side lesion) and 5.03 ± 0.23 (right-side lesion) µg g⁻¹ Cd remained in the lesions for 72 h after the injection (Figure 3F) and that the amount of Cd atoms decreased to 0.916 ± 0.37 (left-side lesion) and 0.983 ± 0.43 (right-side lesion) µg g⁻¹ at 144 h postinjection (Figure S5C, Supporting Information). These results confirmed that QDs accumulate in the lesions, which constitutes the EPR effect.

HAuNS and TNYL-HAuNS had a significantly larger size (up to 40 nm) than QDs (less than 10 nm). The biodistribution of these nanoparticles was also investigated (using indocyanine green, ICG, fluorescence to label molecules) after the intravenous injection of the nanoparticles into the mice with endometriotic lesions. Unambiguous fluorescence signals of both nanoparticles were observed overtime postinjection in the region of the lesions for 144 h, and TNYL-HAuNS displayed stronger fluorescence signals in the lesions than HAuNS (Figure 4A) after 12 h of injection. The fluorescence intensity of HAuNS and TNYL-HAuNS was both decreased from 96 h postinjection (Figure S5B, Supporting Information). Both HAuNS and TNYL-HAuNS accumulated primarily in the liver, spleen, and lesion (Figure 4B,C). TNYL-HAuNS accumulated significantly more in the endometriotic site than HAuNS, which was confirmed by the fluorescence intensity analysis of various tissue and Au element quantitation using ICP-MS. For example, the fluorescence intensities of HAuNS and TNYL-HAuNS were 16.25 ± 2.4 and 23.68 ± 3.2 ID% g⁻¹, respectively, at 72 h postinjection (Figure 4C). The amount of elemental Au in the lesions was 10.35 ± 0.87 µg g⁻¹ at 72 h after the TNYL-HAuNS injection, which was almost two times higher than that after HAuNS injection (5.38 ± 0.14 µg g⁻¹) (Figure 4D). At 144 h after TNYL-HAuNS injection, the amount of Au element in the lesions was 3.35 ± 0.21 µg g⁻¹, which was also much
higher than that after HAuNS injection (Figure S5D,E, Supporting Information). The TEM analysis indicates that both HAuNS and TNYL-HAuNS were distributed throughout the lesions (Figure 4E). The heart, liver, spleen, and lesions were then cut into sections to investigate the distribution of the nanoparticles. The fluorescence signals in the liver and lesions were higher than those in the heart and kidney (Figure 4F), and the fluorescence intensity of TNYL-HAuNS in the lesions was higher than that of HAuNS (Figures S7, Supporting Information). The results demonstrate that the targeted delivery of HAuNS into the endometriotic site can be enhanced via the mediation of the EphB4 receptor.[17]

2.7. Mechanism of Accumulation into the Endometriotic Lesions

The retention of QDs and HAuNS in the endometriotic lesions by the EPR effect was subsequently investigated. After the intravenous injection of QDs, the microvessels...
in the lesion slices were stained dark using CD31 antibody (Figure 5A, yellow arrows). The strong red fluorescence of QDs was observed in the slides, mainly in the vessels of the lesions. Some QDs extravasated from the microvessels and penetrated into a deeper lesion matrix (green arrows), suggesting that the small QDs (less than 10 nm in diameter) could remain in endometriotic lesions through the permeable vessels.\[^{[18]}\] Almost no fluorescence appeared in the lesion slices without QD injection, which served as the control.

Although the sizes of HAuNS and TNYL-HAuNS are more than five times that of the QDs, a strong ICG fluorescence signal of their nanoparticles was observed in the edge and center of endometriotic lesions (Figure 5B), which indicates that HAuNS and TNYL-HAuNS can efficiently accumulate into the lesions after their intravenous injection. Relative to HAuNS, TNYL-HAuNS induced a stronger fluorescence intensity of ICG in the lesions, which is consistent with the results in Figure 4. Furthermore, almost all magenta ICG fluorescence was colocalized with red fluorescence from the staining with anti-EphB4 antibody; thus, TNYL-HAuNS exhibited improved binding with the EphB4 receptor via the mediation of TNYL. Most HAuNS remained around the microvessels after their accumulation in the lesions.

The colocalization parameter ($i_{\text{colocalization}}$) in a selected region of slides (Figure 5C) was further measured using Pearson’s correlation coefficient ($\rho_p$) (Figure 5D), which can range from −1 to 1 as a measure of the correlation between the intensity distributions of various components and was calculated using Metamorph software. The values of 1, 0, and −1 represent perfect correlation, random localization, and perfect exclusion, respectively.\[^{[19]}\] The $\rho_p$ values of the fluorescence signals between EphB4 and CD31 in HAuNS- and TNYL-HAuNS-treated slides were 0.421 and 0.732, respectively, which indicate that the positions of the new vessels were correlated with the EphB4 receptors. The $\rho_p$ values between HAuNS and EphB4 were −0.313 and nearly zero, respectively, which indicates poor colocalization and random correlation. The $\rho_p$ value between TNYL-HAuNS and EphB4 was 0.732, which further demonstrates the high affinity binding between the nanoparticles and the receptors.
The ρp value between ICG and CD31 in the HAuNS-treated slides was 0.041, which indicates a random correlation between HAuNS and microvessels; thus, the nanoparticles were efficiently extravasated from the microvessels. Relative to HAuNS, TNYL-HAuNS had higher correlation with the microvessels (ρp = 0.258), which implies their lower microvessel extravasation. A possible explanation is that the high expression of the EphB4 receptor on microvessels induces a stronger binding between TNYL-HAuNS and microvessels, which obstructs their microvessel extravasation.

### 2.8. Treatment of the Endometriosis Lesions

Saline, HAuNS, and TNYL-HAuNS were intravenously injected into the mice with endometriosis lesions, and NIR laser irradiation was applied with different power levels and durations (Table 1). Representative photographs of the lesions during the treatment process are shown in Figure 6A and Figure S8 (Supporting Information). Figure 6B shows the lesion growth curves in various treatment groups. HAuNS without laser irradiation and PBS affected the lesion growth equivalently, which indicates a notably poor inhibition. For the treatment of HAuNS with NIR laser, a dosage-dependent therapeutic efficiency upon laser irradiation was observed. TNYL-HAuNS with a high laser irradiation dose (2 W cm⁻², 10 min) exhibited a particularly significant inhibition of lesion growth, and the lesion tended toward elimination after one month (Figure S8, Supporting Information). Figure 6C,D shows the inhibition rate of lesion growth by calculating the weight of remained lesions after various treatments. Under the irradiation power of 2 W cm⁻² for 10 min, TNYL-HAuNS caused the strongest lesion growth inhibition (92.7%) relative to HAuNS (77.2%). Mice intravenously injected with 200 μL of sterile saline were treated with laser irradiation (2 W cm⁻², 10 min), and the lesions were photographed after 30 d. It was found that the laser caused minor damage to the lesion and that the lesion grew gradually during the 30 d duration (Figure S9A, Supporting Information). In addition, mice were intravenously injected with 200 μL of sterile saline or HAuNS (2.5 mg Au per mL, twice the dose in the PTA treatment), and the lesions before and after laser irradiation (2 W cm⁻²) were photographed (Figure S9B, Supporting Information). For the saline group, little damage could be observed in the lesion after laser irradiation for 10 min. However, for the HAuNS group, the congestion on the skin of the lesion could be observed after 5 min of laser irradiation, and the damage was more serious when the irradiation time was extended to 10 min. All of the results demonstrated that laser irradiation alone (2 W cm⁻², 10 min) causes little damage to the lesion without HAuNS and that substantial therapeutic efficiency can be attributed to the PTA effect of HAuNS.

The body weight of the mice slightly decreased from day 0 to day 3 in partial nanoparticle-treated groups and then consistently increased in the subsequent period (Figure 6E), which suggests the low systemic toxicity of the treatments. Slices of major organs in various treatment groups showed no noticeable abnormality relative to those in the saline-treated group, which indicates the lack of appreciable organ damage and confirms the limited toxicity of the treatments (Figure S10, Supporting Information).

Slices of lesions in different groups showed a dosage-dependent therapeutic effect from laser irradiation. HAuNS with a higher irradiation condition induced more attenuation at the edge and center of the lesions (Figure 6F,G). TNYL-HAuNS with NIR laser mediation damaged the lesions most and induced the lowest microvessel density (MVD) (Figure 7A–C). SEM images of the endometriotic lesions further demonstrate that the most severely shrunken and disintegrated structure of the lesions was obtained after the treatment with TNYL-HAuNS with the NIR laser (Figure 7D).

The TNF-α and estradiol levels in the serum before and after the treatments were also measured (Figure 7E,F). The TNF-α and estradiol levels clearly decreased for the mice after the treatments with HAuNS or TNYL-HAuNS plus NIR laser and were close to the normal level in healthy mice. Thus, TNYL-HAuNS has an excellent antendometriosis effect with the mediation of an NIR laser.

### 2.9. Discussion

Endometriosis is a chronic gynecological condition that represents a therapeutic challenge. The conventional treatment modalities, namely, hormonal suppression and surgical intervention, often result in serious side effects and high recurrence rates. In this work, we report a new therapeutic strategy for endometriosis that treats the endometriosis cells using NIR-laser-mediated photothermal effects. NIR light can readily penetrate the skin and propagate deep into the tissue because the tissue absorption of light in the NIR region is minimal.[9] Here, HAuNS was used as the photothermal coupling agent because of its high photothermal conversion efficiency under NIR laser irradiation and its favorable biocompatibility.[20]

First, we systematically investigated the characteristic of endometriotic lesions by subcutaneously transplanting the fragments of rat uterus into nude mice. The microstructure and pathological state of the lesions when they grew to 180 mm² on the tenth day after the transplantation (Figure 2A–C) were examined using SEM images (Figure 2D,E), histological analysis (Figure 2F,G) and ELISA
assay (Figure 2H,I), which demonstrated the successful establishment of the endometriotic model. High accumulation of the photothermal coupling agent in the endometriosis site is the key to obtaining a satisfied PTA effect. Then, we investigated the EPR effect in endometriosis lesions using two types of nanoparticles of significantly different size. Our data show that the small nanoparticles (below 10 nm, QDs) and larger ones (up to \( \approx 40 \) nm, HAuNS) can accumulate in endometriosis lesions for at least 144 h (Figures 3 and 4) and further extravasate from the microvessels into the deeper matrix of the lesions (Figure 5). The different biodistribution behaviors of QDs and HAuNS in vivo can be attributed to their different chemical and physical properties. Nanoparticles with the proper size can enter and exit the lesion, and their prevalence in the lesion changes constantly and increases overtime. Smaller QDs move faster than larger one in blood vessels, and it might be possible that smaller nanoparticles preferentially exit the lesion.\(^{[21]}\) The migration rate differs among nanoparticles of different sizes, inducing differential accumulation in the lesions.

Endometriosis tissues are thought to express a high level of neovascularization, similar to cancer.\(^{[13]}\) New vessels are

**Figure 6.** Photothermal effect, irradiation dosage-dependent antilesion efficacy, and histological analysis study of HAuNS and TNYL-HAuNS in vivo. A) Photographs of lesions in endometriosis model mice during the process of photothermal treatment for 30 d. B) The average lesion volumes of each group was monitored over the course of the study (n = 6). C,D) All the lesions (n = 6) were collected and weighed after the mice were sacrificed. E) Monitoring of body weight of the mice. The body weight of the mice was monitored during the entire study to evaluate any acute toxicity caused by the treatment. F) The edge region and G) center region of the endometriotic lesions were stained by H&E and observed under a light microscope. Scale bar: 50 µm. All of the data are represented and analyzed by one-way ANOVA (*\( P \leq 0.05 \); **\( P \leq 0.01 \)).
frequently associated with Eph receptors, which constitute the largest known family of receptor tyrosine kinases and have been reported to control various pathological processes associated with angiogenesis and chronic pain after tissue damage.\[22\] We investigated the expression of the EphB4 receptor in endometriosis tissues using Western bolt and immunohistochemistry and found that EphB4 was highly expressed in the endometriotic lesion and congestive uterus, whereas the expression was negative for the normal uterus (Figure 2J–L). A specific PTA therapy against endometriotic lesions is enabled by the targeted delivery of a photothermal coupling agent into the endometriotic site via the mediation of the EphB4 receptor to further enhance the PTA efficacy and decrease the side effects. Thus, we modified the surface of HAuNS by the conjugation of TNYL peptide, which presented a high binding efficiency with the EphB4 receptors. As expected, TNYL-HAuNS accumulated more in the endometriotic site than HAuNS (Figure 4A–D) and consequently presented the strongest PTA effect under NIR laser irradiation (Figures 6 and 7). Furthermore, we found no detectable damage to normal organs over a 30 d irradiation treatment period after injecting the nanoparticles (Figure S10, Supporting Information). These results indicate that our developed strategy is effective and safe for endometriosis therapy.

A potential limitation of the PTA therapy strategy is the low penetration depth of NIR light. The current protocol enables NIR light to penetrate up to 1–2 cm in soft tissues.\[23\] The therapy in this study is based on subcutaneous endometriotic lesions, and the laser can be easily conducted into the lesions. However, PTA therapy for deep endometriosis lesions should also be feasible. The laser beam can be delivered through fibers inserted into the deep lesions, as in the case of therapy for primary and metastatic liver cancer and lung metastases.\[24\] However, the specific PTA therapy of orthotopic endometriosis lesions mediated by EphB4 should be performed when the uterus is in the normal state but not the congestive state (e.g., menses and estrus) because EphB4 also has a high expression level in the congestive uterus.

3. Conclusion

In summary, we reported a new therapeutic strategy for endometriosis that treats the endometriosis lesions using NIR
laser-mediated photothermal effects. We investigated the EPR effect of two types of nanoparticles with different sizes (QDs and HAuNS) in the lesions. To increase the delivery of HAuNS into lesion cells, the surface of HAuNS was successfully conjugated with the TNYL peptide, which exhibits high-affinity binding with the EphB4 receptor. We found that EphB4 was overexpressed in endometriosis lesions. Our data indicate that both QDs with <10 nm and HAuNS with ≈40 nm diameters could efficiently accumulate in an endometriosis site through permeable vessels and that TNYL-HAuNS accumulate more via the interaction with EphB4. The specific PTA based on TNYL-HAuNS significantly inhibits the growth of the endometriotic volume and induces the atrophy and degeneration of ectopic endometrium with no detectable toxicity to the normal organs. In addition, a significant reduction in MVD was observed, and the morphology of the lesion became looser and disintegrated after the treatment. The level of TNF-α and estradiol also significantly decreased in the endometriotic lesions, which indicates that the treatment enabled a recovery from hormonal imbalance and inflammatory injury. Our work presents an efficient and safe strategy for endometriosis treatment, and is expected to be a valuable reference for further endometriosis therapy.

4. Experimental Section

Reagents and Cell Lines: Cobalt (VI) chloride hexahydrate (CoCl₂·6H₂O), sodium borohydride (NaBH₄), sodium citrate, and chlorauric acid trihydrate (HAuCl₄·3H₂O) were from Thermo Fisher Scientific (Waltham, MA, USA). Cadmium chloride (CdCl₂), sodium tellurite (Na₂TeO₃), 3-mercaptopropionic acid (MPA), polyethylenimine (PEI, M₉₀₀₀), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazole (MTT), and the estradiol ELISA Kit were from Sigma-Aldrich Inc. (St Louis, MO, USA). NaOH, H₂SO₄, and HNO₃ were from Sinopharm Chemical Reagents Company (Shanghai, China). TNYL peptide (sequence: TNYLFSPNGPIA) was from Baiatai Biotechnology Inc. (Guangzhou, China). ICG was from Chengdu Biotech Group, Inc. (Chengdu, China). EphB4 antibody was from Cell Biology of the Chinese Academy of Sciences (IBCB, Shanghai, China). The TNF-α ELISA Kit was from Boster Biological Engineering Co., Ltd. (Wuhan, China). All other chemicals were of analytical grade and used without further purification. Deionized water used in all experiments was prepared using a Milli-Q system (Millipore, Billerica, USA). L929 (mouse fibroblast) cells were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (IBCB, Shanghai, China). The cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO₂.

Synthesis and Physicochemical Characterization of CdTe QDs: MPA-CdTe QDs were prepared as described with some modifications.[25] Briefly, 0.16 mmol of cadmium chloride (CdCl₂) was dissolved in 50 mL of water and transferred into a three-neck flask. Then, 0.85 mmol of MPA and 0.34 mmol sodium citrate were added under stirring, and the pH was adjusted to 11.0 by adding 0.5 mL solution of NaOH (1 mol L⁻¹). The solution was deaerated by N₂ bubbling for 30 min. Then, 2.65 mmol NaBH₄ and 0.04 mmol Na₂TeO₃ were injected into the three-neck flask. After 15 min of vigorously stirring, the solution was refluxed for 30 min to 14 h to control the growth of the nanoparticles of CdTe QDs.[26]

The morphology of the nanoparticles was characterized by TEM (JEM-1230, Japan), and the average size of the CdTe QDs was determined by DLS (Malvern Nano-ZS 90, Malvern, UK). The spectroscopic properties were investigated using an UV–vis absorption spectrophotometer (Hitachi-4100, Japan) and a fluorescence spectrometer (Hitachi-7000, Japan). The fluorescence signal was also examined using a Maestro imaging system (CRI, Inc., Woburn, MA, USA). The photoluminescence QY (Φ₁) of the QDs was measured by comparing the integrated photoluminescence intensity (at 370 nm) of the QDs with that of rhodamine 6G (reference compound), using the following formula

\[
\Phi_1 = \frac{A_{\text{ref}}}{A_1} \left[ \frac{F_{\text{ref}}}{F_1} \right] \left( \frac{n_1}{n_{\text{ref}}} \right)^2
\]

where \(\Phi_1\) and \(\Phi_0\) are the QY of rhodamine 6G and QDs, \(A_{\text{ref}}\) and \(A_1\) are the optical densities of rhodamine 6G and QDs at the excitation wavelength, \(F_{\text{ref}}\) and \(F_1\) are the integral areas under the corrected fluorescence spectrum of rhodamine 6G and QDs, and \(n_1\) and \(n_{\text{ref}}\) are the refractive indices of the solvent for rhodamine 6G (in ethanol, \(n_{\text{ethanol}} = 1.359\)) and QDs (in water, \(n_{\text{water}} = 1.333\)).[27]

Synthesis and Physicochemical Characterization of HAuNS and TNYL-Conjugated HAuNS: HAuNS were synthesized according to the previous approaches.[28] Briefly, 1 mL of (1 mol L⁻¹) cobalt chloride hexahydrate (CoCl₂·6H₂O) was mixed with 4.5 mL of (1 mol L⁻¹) NaBH₄ and 2.8 mL of (0.1 mol L⁻¹) sodium citrate. Cobalt nanoparticles were obtained by reducing cobalt chloride using sodium borohydride. Then, chlorauric acid trihydrate (HAuCl₄·3H₂O) was added into the cobalt nanoparticle suspension, and the gold ions were reduced onto the surface of the cobalt nanoparticles. HAuNS were obtained after the cobalt nanoparticles were oxidized to cobalt oxide by air. The HAuNS were concentrated by centrifugation (10 000 rpm, 10 min).

HAuNS were further modified with the TNYL peptide. Briefly, SH-PeG₅₀₀₀-TNYL was first synthesized according to the previous method[10,15] to obtain TNYL-HAuNS, 50 nmol SH-PeG₅₀₀₀-TNYL and SH-PeG₅₀₀₀ was mixed with 1 mL of HAuNS (5 mg mL⁻¹) and stirred overnight. Then, TNYL-HAuNS were purified by centrifugation and washed with water. The morphology and structure of HAuNS and TNYL-HAuNS were characterized by TEM. The spectra of HAuNS and TNYL-HAuNS were measured using an UV–vis absorption spectrophotometer. The average size was determined using DLS.

Photothermal Conversion Effect: The photothermal conversion effect of HAuNS and TNYL-HAuNS, which was induced by the NIR laser irradiation, was evaluated by exposing the nanoparticle suspensions (Au concentration: 15 μg mL⁻¹) to the emitted radiation of an 808 nm laser at a power density of 1.5 W cm⁻² (Diomed 15 plus, UK). The temperature of the suspension was monitored, using a thermocouple immersed in the suspension, at 30 s intervals for a total of 10 min.

Cytotoxicity Assay: The cell cytotoxicity was measured using an MTT assay according to the manufacturer’s suggested procedures. Mice fibroblast (L929) cells were cultured in a 96-well plate.
at a density of 5 × 10^7 cells per well and treated with different concentrations of QDs, HAuNS or TNYL-HAuNS for 24 h. The data are expressed as the percentage of surviving cells, and reported values are the mean values of six measurements.

**Establishment of Endometriotic Lesion Model:** Female Balb/c nude mice (five to six weeks, 18–20 g) and female Sprague-Dawley rats (seven to eight weeks, 200–240 g) were used in this experiment. They were fed with sterilized water and standard rat chow in a controlled environment (21 °C, 12:12 h light/dark circle). In vivo experiments were performed in compliance with the Zhejiang University Animal Study Committee’s requirements for the care and use of laboratory animals in research. All rats were acclimatized for at least one week before operation, and only the rats that exhibited regular 4–5 d estrous cycles were used. All nude mice were intramuscularly injected with estrogen (Hangzhou First Veterinary Drug Manufacturing Co., Ltd., Zhejiang) for 3 d (one injection per day) before the operation.

The endometriotic lesion model was obtained by subcutaneously transplanting the fragments (~40 mg) of rat uterus into the nude mice. Briefly, the donor rats were sacrificed and their uterine horns were harvested and cut into two equal-sized pieces under sterile conditions. Each piece was minced and placed in 1 mL of Dulbecco’s Modified Eagle Media (DMEM) at 37 °C. Then, 40 mg of uterine tissue was subcutaneously injected into the nude mice. The nude mice were subsequently injected with estrogen three times (one injection per two days). The endometriotic lesions with a volume of 100 mm³ were used for further experiments.

To confirm the endometriosis formation, fresh lesion tissues were cut into 5 μm slides for H&E staining or incubated with the CD31 antibody. Furthermore, the lesions were rinsed with PBS and fixed with 2.5% (v/v) glutaraldehyde for SEM observation (Hitachi S-3000N, Japan).

The levels of TNF-α and estradiol in normal mouse uterus, congestive uterus, and endometriotic lesions were also analyzed using ELISA according to previously reported methods.[29]

**Expression of the EphB4 Receptor on the Endometriotic Lesions:** Normal mouse uterus, congestive uterus, and endometriotic lesions were collected, embedded in Tissue Tek O.C.T. and cut into 5 μm slices for the immunohistochemistry assay. The slices were incubated with the EphB4 primary antibody and, fluorescein isothiocyanate (FITC)-conjugated secondary antibody. The slides were mounted and observed using a confocal microscope (Zeiss, 710, LSM, Germany).

For the Western blot analysis, the tissues were collected with lysis buffer containing a protease inhibitor cocktail and centrifuged at 13 500 rpm. The total protein concentration in the supernatants was quantified using an enhanced bicinchoninic acid (BCA) protein assay kit. Equal amounts of protein from each experimental group were resolved by a 4%–12% SDS-PAGE gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. The EphB4 expression was probed with rabbit anti-EphB4 antibody and antirabbit horseradish peroxidase-conjugated secondary antibody. β-Actin was used as a control to indicate the loading and transfer efficiency. Protein bands were detected using the enhanced chemiluminescence Western blotting substrate (Thermo Scientific). Densitometry was performed using ImageJ Software (http://imagej.nih.gov/ij/).

**In Vivo Biodistribution of QDs, HAuNS, and TNYL-HAuNS in Endometriotic Lesions:** ICG-labeled HAuNS (ICG-HAuNS) and TNYL-HAuNS (ICG-TNYL-HAuNS) were synthesized. Briefly, to synthesize PEI-conjugated HAuNS or TNYL-HAuNS (PEI-HAuNS, PEI-TNYL-HAuNS), PEI (Mw = 2 kDa) was reacted with Traut’s reagent (PEI:TR = 1:5, mol mol⁻¹) and then mixed with HAuNS (or TNYL-HAuNS) in DMF, followed by centrifugation for purification. ICG was mixed with EDC and DMAP (ICG:EDC:DMAP = 1:3:3 mol⁻¹) in deionized water for 1 h. Then, PEI-HAuNS (or PEI-TNYL-HAuNS) was added under stirring for another 24 h to obtain ICG-HAuNS (or ICG-TNYL-HAuNS). Free ICG was removed by centrifugation and washed with deionized water several times.

The mice with endometriotic lesions were intravenously injected with 200 µL of CdTe QDs (0.4 mg mL⁻¹), ICG-HAuNS (1.25 mg Au per mL) or ICG-TNYL-HAuNS (1.25 mg Au per mL). The real-time distribution of nanoparticles in the mice was determined by a Maestro imaging system (CRI, Inc., Woburn, MA, USA). The mice were sacrificed at 72 h postinjection, respectively. The tissues (heart, liver, spleen, lung, kidney, and lesion) were collected, and their fluorescence intensity was quantified using the system. Slides of the tissues were also prepared for observation with a Zeiss fluorescence microscope.

To investigate the intradistribution of CdTe QDs in the endometriotic lesions, neovascular cells in the lesion slices were stained with CD31 antibody and observed using a light microscope. Then, the distribution of QDs in the lesions was observed using a fluorescence microscope. To evaluate the specific-binding effect of TNYL-HAuNS in the lesions, neovascular cells and EphB4 receptor in the lesion slices were stained with FITC-conjugated CD31 antibody and Cy3-conjugated EphB4 antibody, respectively, and observed by fluorescence microscopy.

For ICP-MS, the mice with lesions were injected with 200 µL of CdTe QDs (0.4 mg mL⁻¹), ICG-HAuNS (1.25 mg Au per mL) or ICG-TNYL-HAuNS (1.25 mg Au per mL). 72 and 144 h after injection, the heart, liver, spleen, lung, kidney, and lesions were weighed and freeze-dried. The samples were digested with 4 mL of aqua regia. The solution was evaporated, and the precipitate was suspended in an aqueous solution that contained 1.5% HCl and 0.5% HNO3, and centrifuged at 10 000 rpm for 10 min. The Cd or Au content in the supernatant was analyzed using an ICP-MS (Elan DRC II PerkinElmer, Waltham, MA, USA).

The lesions were fixed with 2.5% glutaraldehyde in PBS (pH 7.0) for more than 4 h, washed three times with PBS, postfixed with 1% OsO4 in PBS (pH 7.0) for 1 h and washed another three times with PBS. Then, the specimens were dehydrated by a grade series of ethanol and absolute acetone, infiltrated, and embedded. From the embedded lesions, ultrathin sections were cut, mounted on copper grids, stained with uranyl acetate and alkaline lead citrate, and observed by TEM.

**Efficacy Assessment Using the Endometriosis Model:** The mice with endometriotic lesions were randomly divided into nine groups of six mice in each group. As a negative control, the mice in group 1 were given 200 µL of sterile saline with intravenous injection on day 0. The mice in groups 2–8 were given 200 µL of HAuNS (1.25 mg Au per mL) on day 0. They were further irradiated with the NIR laser on days 2 and 4 after the injection (Table 1). The mice in group 9 were given 200 µL of TNYL-HAuNS (1.25 mg Au per mL) on day 0 and irradiated with the NIR laser (2 W cm⁻², 10 min) on days 2 and 4. The endometriotic lesion volume was measured using a digital caliper, and pictures of the lesions were captured. The endometriotic lesion volume and animal bodyweight...
were measured every 3 d for 30 d after the injection. The endometriotic lesion volume was determined as (length × width²)/2. The endometriotic lesion growth inhibition (%) was calculated as (1 – average lesion volume of treated group/average lesion volume of control group) × 100%. Mice intravenously injected with 200 µL of saline were treated with laser irradiation (2 W cm⁻², 10 min), and the lesions were photographed after 30 d. In addition, mice were intravenously injected with 200 µL of saline or HAuNS (2.5 mg Au per mL), and the lesions before and after laser irradiation (2 W cm⁻²) were photographed.

The mice were sacrificed on day 30 after the injection, and the lesions and other organs were collected. The fresh lesion tissues were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5 µm slides, and stained with CD31 antibody to determine the MVD of the endometriotic lesions. Other organs were fixed for H&E staining and examined under a light microscope.

The lesions of the mice in groups 1, 8, and 9 were further observed by SEM as previously described. The serum of normal mice and the mice in groups 1, 8, and 9 was collected and analyzed by the level of TNF-α and estradiol using ELISA according to the aforementioned method.

Statistical Analysis: Comparative analysis of the difference between groups was performed using the two-slide Student's t-test (Excel software, Microsoft). A statistically significant difference was determined at p < 0.05. Values were expressed as the mean ± SD, as stated in the figure legends.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
The research was supported by the National Nature Science Foundation of China (81373348 and 81573365).


