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A smart phase transitional and injectable DOX/PLGA-Fe implant for magnetic-hyperthermia-induced synergistic tumor eradication

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ABSTRACT

Magnetic hyperthermia ablation is a new and minimally invasive modality for localized tumor removal. However, an inadequate ablation dosage can leave a residual tumor or cause a variety of complications. In addition, commonly used magnetic nanoparticles can easily escape from the tumor tissue, which presents potential safety problems. In this study, a smart phase transitional and injectable implant based on biocompatible poly lactic-co-glycolic acid (PLGA) implant incorporating magnetic material (Fe powder) and anti-cancer drug (doxorubicin (DOX)) was developed. The magnetic-induced hyperthermia and release efficiency of DOX was evaluated in vitro. Drug release can be controlled under external alternating current magnetic field (AMF). The results of the in vivo tumor therapeutic efficacy showed that when exposed to external AMF, this smart injectable DOX/PLGA-Fe implant could converse magnetic energy into heat and accelerate the release of DOX, which leads to increasing the temperature to achieve tumor coagulative necrosis and accelerating the release of DOX to enhance residual tumor apoptosis. Furthermore, there was no leakage of magnetic material, as demonstrated using real-time ultrasound (US) and computerized tomography (CT) imaging, realizing the guidance and monitoring of tumor therapy. In conclusion, this smart phase transitional and injectable implant DOX/PLGA-Fe has the ability to improve the efficiency of this newly developed minimally invasive magnetic ablation of tumor treatment technique, and will provide a new avenue of developing minimally invasive synergistic tumor therapy.

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for highly efficient in vivo magnetic hyperthermia therapy of tumor 
[17]. Upon contact with the surrounding aqueous environment, 
solvent (NMP) diffuses can precipitate hydrophobic PLGA, 
and make it restricted efficiently when a solid or semisolid state 
is formed; this is so called mechanism of solvent-removal precipi-
tation. As a result, this smart injectable NMP/PLGA-Fe can change 
from liquid phase to solid phase in situ after a minimally injection 
into tumor tissue and efficient restricted in tumor area to achieve 
local tumor ablation by magnetic-hyperthermia. Unlike traditional 
nanomagnetic fluid materials with low concentration in tumor 
region, or lack of degradation for ferromagnetic thermo seeds, this 
on-demand NMP/PLGA-Fe traps magnetic particles tightly and 
with good biodegradable properties of PLGA and Fe [18–20]. This 
highly efficient and versatile approach has a bright future in 
magnetic-hyperthermia-induced ablation when applied for mini-
mally invasive regional hyperthermia in tumor treatment.

However, like all minimally invasive techniques, magnetic- 
induced ablation for tumor based on this smart injectable system 
(NMP/PLGA-Fe) also has the drawbacks of residual tumor leading 
to the tumor recurrence and normal tissue damage. Synergistic ther-
mal–chemotherapy of cancer with high therapeutic efficacy, with 
the addition of hyperthermia, tumor cells are more sensitive to 
cytostatic drugs, thus, tumor recurrence can be inhibited [9]. Previ-
ous research showed that combined local hyperthermia and 
intravesical chemotherapy was efficient for patients with bladder 
cancer; 80.9% of patients did not experience tumor recurrence 
[21]. Min Jeong Jeon showed that the in situ injection of ferrucarbo-
tran conjugated with DOX into an hepatic cellular cancer (HCC) 
model combined with magnetic-induced hyperthermia ablation 
showed substantially improved therapeutic efficiency compared 
with either alone [22], Feng Zhang showed that a combination of 
chemotherapy and radio frequency hyperthermia can result in 
lower mean levels of cell proliferation and lower percentages of 
relative tumor volume than either treatment alone [23].

Inspired by the above injectable smart phase-transition implant 
and the potential of a synergistic therapeutic strategy based on 
combined chemohyperthermia systems, we sought to develop a 
smart injectable and DOX-loaded multifunctional liquid–solid 
phase-transition material (DOX/PLGA-Fe) that can utilize both 
ultrasound (US) and computed tomography (CT) dual-mode guided 
and monitored chemohyperthermic synergistic therapy. This 
smart injectable DOX/PLGA-Fe implant can generate heat when 
exposed to AMF, which can increase the temperature to achieve 
tumor coagulative necrosis and accelerate the release of DOX to 
enhance the apoptosis of residual tumors (Fig. 1). In addition, ther-
apetic process can easily repeated after only once invasive injec-
tion of this material due to the in situ liquid–solid phase 
transformation in the tumor tissue. The biocompatible DOX/
PLGA-Fe can completely eliminate the tumor without recurrence 
and improve the comfort and compliance for patients, which pro-
motes its further clinical translation.

2. Materials and methods

2.1. Preparation of DOX/PLGA-Fe

The preparation of DOX/PLGA-Fe was similar to the previous 
research [17]. Typically, the DOX (Sigma–Aldrich) was dissolved 
into N-methyl pyrrolidone (NMP, Sigma–Aldrich), and then PLGA 
(0.55 g/ml, Ananti Inc.) was added into the above solution to form 
DOX/PLGA. After storage in an incubator at 37 °C overnight, the Fe 
powder (Sharp Company) were dispersed into this DOX/PLGA solu-
tion via mechanical stirring. The ultimate concentration of DOX 
was 1%.

2.2. In vitro magnetic-hyperthermia-induced hyperthermia 
evaluations

For the in vitro assay, three types of liquid DOX/PLGA-Fe (50 μl) 
containing different amounts of Fe powder (1%, 10%, and 20%) were 
put into saline solution (1.5 ml) in Eppendorf tubes (2 ml). The 
Eppendorf tubes were put in the center of the electromagnetic 
induction heating coil of a homemade magnetic hyperthermia ana-
lyzer (frequency: 626 kHz, output current: 28.6 A, coil diameter: 
3 cm). After exposure to AMF, infrared thermal images of the 
Eppendorf tube were taken every 10 s over a span of 110 s. The 
Eppendorf tube containing only saline solution was used under 
the same conditions as the blank control. The temperatures of 
the saline solution were analyzed using the thermal images.

2.3. Ex vivo bovine liver magnetic-induced ablation evaluations

For this study, fresh ex vivo bovine liver (2 cm × 2 cm × 2 cm) 
was used. Liquid DOX/PLGA-20% Fe was injected into the middle 
of the excised organization block via syringe. After the liquid 
DOX/PLGA-20% Fe transformed into a solid (approximately 
1 min), the organization block containing solid DOX/PLGA-20% Fe 
was put into the center of the electromagnetic induction heating 
coil and was exposed to AMF for 2 min, 3 min and 5 min, respec-
tively. Afterward, temperature variations in the organization block 
were recorded every 10 s. Two items were observed at 2 min, 
3 min, and 5 min: (1) US images of the organization block prior 
to and after ablation and (2) the coagulative necrosis area after 
ablation. Experiments for each group were performed in triplicate.

2.4. In vitro and ex vivo bovine liver magnetic-enhanced release of 
DOX

An in vitro magnetically enhanced release experiment was per-
fomed to assess the DOX release behavior and efficiency in various 
ph levels when exposed to AMF. Liquid DOX/PLGA-20% Fe was pre-
pared. A total of 50 μl liquid DOX/PLGA-20% Fe was dropped in a 
diagnosis bottle (MWCO: 8000–14000), with 1 ml phosphate-
buffed saline (PBS, pH 5.0 and pH 7.4) and then placed in a 45-
ml centrifuge tube with an additional 29 ml PBS of the same pH. 
An incubated shaker was then used to perform the release at 
37 °C and 100 rpm. Samples of 1 ml were taken at 30 min intervals, 
and the corresponding solution of equal volume was added back 
to maintain the total volume of the release medium of 30 ml. At time 
points of 0.5 h, 2 h, 6 h and 21 h, the centrifuge tube was exposed 
to AMF for 10 s. The efficiency of DOX release was determined 
using ultraviolet–visible (UV–Vis) spectrophotometry by detecting 
the amount of DOX in each sample. Then, the accumulative ratios 
of the released DOX were calculated as a function of time. The DOX 
release of PBS at pH 5.0 and pH 7.4 without exposed to AMF were 
assessed with the same method to establish control groups.

The magnetic-enhanced release and distribution of DOX was 
evaluated in ex vivo bovine liver. After an injection of DOX/PLGA-
20% Fe and AMF application for 2 min, 3 min, and 5 min, tissues 
1 cm distal from the center of the ablation area were collected 
for snap-frozen sections. The fluorescence intensity of the inher-
ently fluorescent DOX was then observed via inverse fluorescent 
microscope.

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Fig. 1. Schematic of the smart injectable multifunctional implants for tumor therapy. This smart injectable multifunctional implant (DOX/PLGA-Fe), with ability of liquid–solid phase transformation responsive to body fluid, incorporating magnetic material (Fe powders) and anti-cancer drug (DOX) was constructed, and can be detected under US and CT for dual-mode guiding and monitoring magnetic thermal–chemo tumor therapy.
emagnetic energy into heat under AMF. As illustrated in Fig. 3A, with increasing time under AMF, the color of images in PBS without DOX/PLGA-Fe had barely changed. With the increase of Fe powder concentration, the images of DOX/PLGA-Fe showed the highest temperature among them (DOX/PLGA-1% Fe, DOX/PLGA-10% Fe, DOX/PLGA-20% Fe). The corresponding time–temperature curves acquired from these images were described in Fig. 3B. All of the temperatures substantially increased with time except the PBS group (control). The increased temperature ($\Delta T/\degree C$) of DOX/PLGA-1% Fe, DOX/PLGA-10% Fe and DOX/PLGA-20% Fe groups after 110 s under AMF were $12 \pm 1.8 \degree C$, $30 \pm 1.5 \degree C$ and $47 \pm 1.2 \degree C$, respectively. With the increasing mass of Fe powder, the temperature showed linearly increase exposed to AMF, demonstrating good magnetic-thermal conversion properties of Fe powder and no significant temperature effect of DOX/PLGA exposed to AMF.

### 3.1.3. Ex vivo bovine liver magnetic-induced ablation

In order to further investigate the magnetic-thermal conversion properties of DOX/PLGA-Fe, ablation efficiency in ex vivo bovine liver was assessed. DOX/PLGA-20% Fe was chose to be the smart injectable system to maximize the high enough temperature for the coagulative necrosis of tumor cell. Thermal images of ex vivo bovine liver without/with 50 $\mu$L DOX/PLGA-20% Fe demonstrated that only the tissue with the magnetic material could be ablated when exposed to AMF. The temperature of bovine liver with injection of DOX/PLGA-20% Fe increased $35 \pm 3.2 \degree C$ under AMF for 110 s, while the temperature of bovine liver as the control group showed no difference during the process, which consistent with the result of DOX/PLGA-Fe in PBS solution (Fig. 4A and B). As shown in Fig. 4C, the ablation area increased from $7.03 \pm 0.76 \text{ mm}^2$ for 2 min to $13.56 \pm 1.75 \text{ mm}^2$ for 5 min under...
AMF, providing guidance for an appropriate ablation dose in further in vivo experiments at some extent. Furthermore, as shown in Fig. 3, after injection of DOX/PLGA-20% Fe, the ultrasound gray value of injection site had great increase due to the liquid–solid phase transformation of DOX/PLGA-20% Fe, indicating DOX/PLGA-20% Fe can be a good ultrasound contrast agent. Moreover, when the bovine liver with injection of DOX/PLGA-20% Fe exposed to AMF, the ultrasound signal intensity of ablation area was high echogenic, which can monitor the therapeutic process of tumor.

3.2. In vitro and ex vivo bovine liver magnetic-enhanced release of DOX

The biocompatible injectable implant, with liquid–solid phase change responsive to aqueous solution, can be a smart drug carrier. As shown in Fig. 5A, DOX and Fe powder dispersed in the NMP/PLGA to form the DOX/PLGA-Fe, which can turn to solid when contacting water. Then, the release profiles of DOX/PLGA-20% Fe at different pH with/without exposed to AMF were studied. As shown in Fig. 5B, there was no significantly difference between the DOX release ratio in pH 7.4 PBS and pH 5.0 PBS, which was not exposed to AMF heating (p > 0.05). It is worth noting that the release percentage increase from ~50% to ~83% with additional AMF at pH 5.0, which indicated that drug release, can be controlled by AMF. Moreover, with the increase of time exposed to AMF, red fluorescence signal, associated with the DOX molecule, enhanced distinctly, consistent with the above result. DOX release and diffusion can be accelerated, which was benefit for increasing the therapeutic efficacy of chemotherapy. The thermal stability of DOX was also confirmed in the high temperature. Fig. 5D showed the OD values @ 808 nm of DOX aqueous solution at 80 °C for 15 min and 30 min were the same as that of the initial DOX, proving DOX has good thermal stability in high temperature.

3.3. In vivo thermal and US/CT imaging for tumor

Inspired by the excellent magnetic-thermal conversion properties and its characteristic of liquid–solid phase transformation responsive to body fluid, thermal imaging, US imaging and CT imaging for DOX/PLGA-20% Fe were performed. The thermal images showed that the temperature of tumor changed in the process with injection of DOX/PLGA-20% Fe exposed to AMF (Fig. 6A). The temperature of tumor with injection of DOX/PLGA-20% Fe exposed to AMF reached to 52 ± 1.5 °C in 110 s, which was higher than the reported temperature of 46 °C for tumor coagulative necrosis [24]. However, the temperature of the tumor without DOX/PLGA-Fe (as the control group) only increased by 1.2 ± 0.3 °C (to a total of lower than 38 °C) (Fig. 6B). DOX-PLGA/20% Fe could change acoustic environment due to the physical property of Fe powder, indicating it is visible under US guiding. As shown in Fig. 6C, tumor with infection of DOX-PLGA/20% Fe had a substantially stronger echo signal than the control group. In addition, the transverse section CT images in Fig. 6C showed that the intensity of tumor with DOX-PLGA/20% Fe injection was significantly brighter than the control group due to the high density of Fe. The 3D reconstruction of CT images also showed the precise location of the DOX-PLGA/20% Fe in the tumor region (Fig. 6D), demonstrating the ease of detecting DOX-PLGA/20% in vivo and the ability to prevent safety problems resulted from leakage. Thus,
355 DOX/PLGA-20% Fe can be visible under US and CT guiding and monitoring.

3.4. In vivo magnetic-induced tumor eradication efficiency evaluation

The excellent in vitro experiment results of DOX/PLGA-20% Fe derived us to investigate its application in vivo. It is well known that one of the drawbacks of the sole chemotherapy is the residual tumor causing the recurrence [8]. In addition, NMP and PLGA with little/no toxicity are biocompatible in the medical application. Additionally, the cytotoxicity assay for normal cell in Fig. S1 also demonstrated the biocompatible property in normal tissue. No significant cytotoxicity of PLGA-Fe was observed for cells, but the decline of cell viability of group DOX/PLGA-Fe was owing to the toxicity of DOX for cells. Furthermore, there was no significant temperature effect of DOX/PLGA exposed to AMF. Therefore, the control group (without treatment), DOX/PLGA, PLGA-20% Fe with AMF (PLGA-20% Fe + AMF) and DOX/PLGA-20% Fe with AMF (DOX/PLGA-20% Fe + AMF) were investigated for high efficient therapeutic therapy of tumors (n = 20). Compared to the control groups and DOX/PLGA, tumors in PLGA-20% Fe + AMF group were ablated by the sole magnetic hyperthermal therapy in some extent with residual tumor. As shown in Fig. 7A and B, tumors of mice in DOX/PLGA-20% Fe + AMF group not only were ablated after 4 days treatment, but eliminated after 5 days. In addition, there were no any noticeable changes of the body weight of mice in all the three groups during the treatment (Fig. 7C). The microscopic structure of the partially ablated tumor showed a disordered cell structure with inhomogeneous cell sizes and shapes and a darkly stained nucleus. Moreover, the cytoplasm was stained a uniform red in the coagulated tumor tissue. The edge of the coagulated tissue could be easily observed under a microscope (Fig. 7D).

The immunohistochemistry staining results showed the apoptosis tumor cells (the positively brown stained cells in the TUNEL
387 images) in the PLGA/DOX-20% Fe with AMF group was significantly higher than the other control groups, and the percentage of proliferating cells (the positively brown stained cells in the PCNA images) in the PLGA/DOX-20% Fe with AMF group was significantly less than the other control groups (Fig. 8A). The related AI value in the PLGA/DOX-20% Fe with AMF group was significantly higher than the other groups, and its PI value was the lowest among all groups (*p < 0.05, Fig. 8B and C).

4. Discussion

In this study, we developed an injectable, liquid to solid phase transitional and DOX-loaded material, the DOX/PLGA-Fe, for the magnetic thermoablation treatment of tumor, and tested it ability in the imaging-guided chemo-hyperthermal synergistic therapy. As a novel material, the safety issue should be considered. At the design stage, we selected the major components of the material with good safety record. The in situ forming implants (ISFs) using PLGA has been reported as a biocompatible and safe drug delivery agent both in vitro and in vivo [5]. And the PLGA ISF containing iron powder (PLGA-Fe ISF), which could achieve complete localized cancer regression via magnetic hyperthermia, was also reported as biocompatible and safe material [17]. In this study, the cytotoxicity assay for normal cell in Fig. S1 also demonstrated the biocompatible property in normal tissue. While the long time degradation needs to be studied in future.

Like the other thermal ablation techniques, such as RF ablation and microwave ablation, the magnetic thermal ablation also faces the same challenging, the tumor recurrence or residual tumor after thermal ablation. The combination of chemotherapy with thermal ablation is an alternative choice. In this study, the DOX was selected based on the following consideration. DOX is one of the most commonly used chemotherapy drugs for many malignant tumors [25–27]. And the DOX-loaded in situ implants could achieve local drug release with a faster drug release rate in stiffer ablated tumor tissues [5,27,28]. Furthermore, the inherent fluorescence of DOX can also provide information on drug distribution after injection, and its thermal stability also guarantees the efficiency of local chemotherapy [25].

To develop the injectable and liquid to solid phase transitional DOX/PLGA-Fe material, the PLGA and the solvent (NMP) were selected. Upon contacting with the surrounding aqueous environ-

Fig. 7. In vivo magnetic thermal therapy and chemotherapy for tumor. (A) Macroscopic tumor model at pre-treatment, as well as 4 and 8 days after ablation (yellow arrow: tumor change in each group; Group A: control; Group B: DOX/PLGA; Group C: PLGA-20% Fe + AMF; Group D: DOXPLGA-20% Fe + AMF). (B and C) The curve of the relative tumor volume and body weight with time prolonging. (D) The microscopic structure of each group (scale bar: 200 μm, HE staining, 100× magnification; red dotted line: the edge of ablation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
ment, the rapid diffusion of NMP inside the aqueous environment can precipitate hydrophobic PLGA to form a solid implant immediately. Thus the Fe powder and the DOX molecule were trapped inside the solid implant accordingly. And the subsequent solidification of the inner part was continuous [17]. Along with diffusion of solvent, the DOX was reported to have the same elution kinetic from the in situ implant [29,30]. In this study, we found the release percentage and the release rate of the DOX was dramatically higher in the exposure to AMF group than the control group without AMF exposure.

In this study, we distributed the DOX inside the material aiming to prevent treat of the possible tumor recurrence or residual tumor. While how the DOX affecting the heating efficiency of the material is a raised concern. Our preliminary data showed the SAR values for 1.5 g DOX/PLGA-20% Fe and 1.5 g PLGA/20% Fe3O4 were 77.05 ± 0.52 W/g and 76.30 ± 0.24 W/g respectively (p > 0.05), which implicated the thermal efficiency of the new system is similar to that of the PLGA/Fe3O4. Furthermore, for this chemotherapy-hyperthermic synergistic therapy, the hypoxia and low pH state needs to be considered. It was reported that the hypoxia and low pH value of the tumor center makes tumor cells more sensitive to hyperthermia and less sensitive to chemotherapy, while the tumor cells in the periphery are sensitive to chemotherapy [31]. In this study, we found that the release percentage of the DOX was significantly higher in the PBS with a pH value of 5.0 than in the PBS with pH value of 7.4, which confirmed the low pH value of the tumor center induced an increased DOX release percentage [32].

To meet the needs of local chemotherapy to reduce the severe side effects of chemotherapy drugs, and to improve the treatment efficiency of the in situ forming drug delivery systems is of importance in the future clinical translation. It was reported that hyperthermia combined with chemotherapy could produce a synergistic effect and make the tumor cells more vulnerable to the cytotoxicity caused by anti-cancer drugs, and this combination could also induce higher levels of drug accumulation in tumor cells [24]. The development of the DOX/PLGA-Fe materials meet the needs of local chemotherapy vehicle to reduce the severe side effects of chemotherapy drugs, and also improved the treatment efficiency of the in situ forming drug delivery systems by the adding value of magnetic hyperthermia. And Figs. 7 and 8 also confirmed the synergistic treating efficiency.

Finally, for the clinical translation in future, the magnetic coil size is a raised concern. It was reported that the induction heating efficiency is positively correlated with induction frequency, energy power and the amount of Fe, and negatively correlated with the coil size [33–36]. To increase the power is relatively easier, while the increase of the coil size will lower the induction frequency, thus it is challenging to increase the whole induction heating efficiency for small amount of the DOX/PLGA-Fe material using big size coil. While a big size coil is needed for human body application, luckily, with the rapid development of magnetic engineering, researchers have developed some equipments with varies coil size which can meet the needs of heating efficiency for clinical translation. The machines have been employed in the human brain and prostate cancer hyperthermia therapy [37,38] and human solid tumor treatment [39]. Thus, such a powerful modality for tumor eradication based on DOX/PLGA-Fe still has the application prospect for magnetic induced hyperthermia.

Although we have proved the efficiency of this chemotherapy-hyperthermal synergistic therapy in this study, the limitations for this study still needs to be considered and to be improved in a further study. First, the temperature of the region of interested did not reflected in thermal images. We tried the internal temperature measurement using a method based on the changes of the ultrasound echo intensity, while this method was affected by many factors and the results varied significantly. The other temperature measurement method, the MR thermometry, has shown an encouraging prospect, while it is not ready for in vivo application either since in referenceless MR thermometry, large susceptibility change can affect polynomial model fitting and result in large temperature error. Recently, Xin Liu et al. proposed a method to improve the accuracy of referenceless MR thermometry method by excluding the regions with large susceptibility artifact automatically based on the local field map derived from the projection onto dipole fields method [20]. This new method has been validated in the precise and real-time intra-procedural evaluation of temperature increases within treatment areas and surrounding tissues in an ex vivo bovine liver study. We would expect a real time monitoring of tumor internal temperature changes using this new method in the near future. Second, the result showed that the temperature of the tumor without DOX/PLGA-Fe was increased 4.8 ± 0.3 ºC, not 0 ºC as expected. The reason might owe to the limitation of the magnetic induction heating equipment since the
coils, in which the mice were put, generated some heat to increase the surface temperature.

5. Conclusion

In conclusion, this study developed an injectable and DOX-loaded multifunctional liquid for solid phase transitional magnetic material (DOX/PLGA-Fe) that can achieve US and CT dual-mode imaging-guided chemo-hyperthermic synergistic therapy and significantly improve the efficiency of a new minimally invasive magnetic ablation technique in tumor treatment.

Competing interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.09.037.

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