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Laser-activated PLGA theranostic agents for cancer therapy in vivo

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***Abstract*— Poly (lactide-co-glycolic acid) (PLGA) particles are biocompatible FDA approved materials that can be used for diagnostic and therapy applications. Micron-sized PLGA particles were synthesized with gold nanoparticles and DiI dye within the PLGA shell, and perfluorohexane liquid (PFH) in the core. The liquid core is activated upon laser irradiation, resulting in a rapidly expanding microbubble; damage to surrounding cells and tissue can occur. The effect of the particles for anti-cancer therapy was examined in rabbits with metastasized squamous carcinoma in the lymph nodes. After laser irradiation, decreased blood perfusion and necrotic regions were observed for rabbits treated with the PLGA particles compared to the control. Electron microscopy and histology confirmed damage within the lymph nodes; disrupted cells were observed throughout, slowing the tumor growth rate. This study demonstrates the capability of PLGA particles containing PFC liquids as theranostic agents in-vivo.**

I. Introduction

Perfluorocarbon (PFC) liquids are FDA approved biomaterials that can be made into nano and micron-sized emulsions [1], [2]. The emulsion can be activated (core vaporized to gas) when acoustically insonified using a process called acoustic droplet vaporization (ADV) [3]–[7]. The potentially violent expansion can be used for cancer therapy via targeted drug delivery and mechanical cancer cell destruction. The PFC emulsions can also be activated via laser irradiation, using a method called optical droplet vaporization (ODV) [8]–[10]. Since PFC liquids have negligible absorption in the 400-1000 nm wavelengths, optically absorbing materials such as gold nanoparticles and dyes are incorporated into the particles to facilitate vaporization. Liquid emulsions use a soft shell such as lipids or albumin to encapsulate the PFC, which limits stability. The emulsions may vaporize spontaneously, resulting in vessel occlusion [11]. Advantages to PLGA materials is that the hard PLGA shell limits spontaneous vaporization, and dyes or particles can be embedded within the shell.

PLGA particles containing perfluorohexane (PFH, C_6F_{14}) in the core and DiI dye and gold nanoparticles (AuNPs) within the shell were investigated as theranostic agents in-vivo. At low laser energies, the PLGA particles will emit a photoacoustic wave but will not vaporize [12], [13]. Thus they can be used as photoacoustic contrast agents to ensure localization within the target area. The laser energy could then be increased to activate the particles, achieving the therapeutic effect via localized drug delivery and/or mechanical cell disruption. The resulting microbubbles can enhance ultrasound imaging. The particles can be used in several capacities: 1) as a photoacoustic contrast agent to confirm delivery of the particles to the targeted area, 2) activated for anti-cancer therapy, 3) then as an ultrasound contrast agent to confirm that vaporization occurred in the targeted region.

In this study, the the PLGA particles containing PFH and AuNPs/DiI dye were investigated first in a gel phantom model to confirm vaporization and optimize parameters. Then the particles were tested in-vivo in rabbit metastatic lymph nodes to demonstrate the particle effectiveness as an anti-cancer treatment.

II. Methods

A. PFC particle preparation

DiI dye (1 mg) and gold nanoparticles (AuNPs, 1 mg) were incorporated into PLGA particles using a double emulsion (water/oil/water) evaporation process. DiI and AuNPs were added to CH_2Cl_2 dissolving PLGA (100 mg) agents, PFH added and then the solution was emulsified using an ultrasonic probe. The resulting solution was poured into PVA solution and homogenized for the second emulsion. The emulsion was then mixed with isopropanol and magnetically stirred for 2 h to extract the CH_2Cl_2. The solution was then centrifuged at 3500 rpm for 3 min. The supernatant was discarded and the precipitate was washed with deionized water. PLGA/PFH particles not containing DiI and AuNPs were prepared as a control. The size distribution, and morphological and structural characterization were estimated by a scanning electron microscope (SEM) and a transmission electron microscope (TEM).

B. *Vaporization and ultrasound imaging measurements using a gel phantom model*

The suitability of PLGA/PFH particles containing DiI/AuNPs was examined as ultrasound contrast agents. A 1% agar gel phantom with a central void created by inserting an eppendorf tube into the center during gel formation was made. A solution containing 100 μg/mL PLGA particles in saline were inserted into the void, then irradiated using a therapeutic laser (Nd:YAG Q5, Leifei Shi, Beijing, China) at 120 mJ. During laser irradiation, the samples were scanned using a contrast enhanced ultrasound imaging (CEUS) and conventional B mode imaging using a linear probe that transmits and receives center frequencies of 5.0 and 12.0 MHz.

C. *Animal Studies*

All in-vivo studies were performed according to a protocol approved by animal ethics committee of Chongqing Medical University. A total of 15 rabbits (2.0-2.5 kg, New Zealand White Rabbits) were used. They were anesthetized with a 3% sodium pentobarbital (0.8 mL/kg) injection via ear vein. The hind legs were depilated with 8% sodium sulfide. Then 1 mL of VX2 squamous carcmina cell tissue suspension was inoculated into the hind legs via injection.

After three weeks, the rabbits were divided into three groups: Group 1 was used an a control using saline injection only, group 2 was injected with the pure PLGA/PFH particles (not containing DiI/AuNPs), and group 3 was injected with PLGA/PFH particles containing DiI/AuNPs. All animal experiments and procedures were performed under complete anesthesia. A seven-day treatment protocol was initiated. Prior to the first treatment, both CEUS and B-mode ultrasound imaging of the lymph nodes were obtained. Then each rabbit in each group received a 2 mL injection of particle solution or saline. The area was massaged for 5 min to stimulate movement into the lymphatic system. the lymph node was irradiated for one minute using a laser with an energy output of 140 mJ. Ultrasound imaging followed by particle injection was then performed every 2 days (on days 3, 5) then on day 7, the animals were sacrificed and the lymph nodes examined with H&E and PCNA-marker.

III. Results and Discussion

A schematic of the PLGA particles are shown in figure 1A, and SEM images of the PLGA/PFH particles are shown in figure 1B-C. The average particle size was 2.5 ± 0.8 μm. The theranostic agents developed can be used as: 1) photoacoustic contrast agents to indicate if the agent has reached the target, 2) therapeutic agents for anti-cancer therapy, and 3) ultrasound contrast agents to validate the therapeutic delivery after vaporization has occurred.

The potential of the PLGA particles loaded with DiI/AuNPs as ultrasound contrast agents was assessed using an agar gel model. A solution of 100 μg/mL PLGA particles in saline was added to the central void. Measurements using standard B-mode ultrasound imaging and contrast-enhanced ultrasound (CEUS) were performed on three different experiments: A) A control containing only saline with no PLGA particles, B) PLGA particles without DiI/AuNPs, and C) PLGA particles loaded with DiI/AuNPs. No change in the ultrasound images before and after laser irradiation was observed for groups A and B (figure 2A). A significant increase in the ultrasound contrast was observed for group C (figure 2A). These measurements show that the PLGA particles containing DiI/AuNPs were vaporized, resulting in a solution of highly echogenic microbubbles. The PLGA particles without DiI/AuNPs, and the saline solution did not have an increase in ultrasound contrast.

The PLGA particles were then investigated as anti-cancer therapy agents in animal models containing metastatic tumors in the lymph nodes. As with the gel phantom measurements, three groups were tested: A) A control containing only saline with no PLGA particles, B) PLGA particles without DiI/AuNPs, and C) PLGA particles loaded with DiI/AuNPs. On day one, prior to therapy, ultrasound B-mode images of the lymph node and a blood perfusion map to acquired measure a baseline (figure 3A). After imaging, the rabbits were injected with the particle solution and irradiated. After two days, ultrasound images and blood perfusion maps were recorded, and then the PLGA particles were injected and irradiated again. This process of ultrasound imaging then laser irradiation was performed on days 1, 3 and 5. A final ultrasound image was recorded on day 7, then the animals were sacrificed and tumors excised.

In group A and B, an increase in lymph node size over the 7-day treatment was observed, with an average volume growth rate of 69.5% and 70%, respectively. In group C, the lymph node growth rate was 38.2%, which was significantly lower than the control groups ($p<0.05$, figure 3B). Additionally, blood perfusion within the lymph node was lower on day 7 compared to day 1. No changes in body weight for any group were observed during the treatment.

The tumor lymph nodes were sectioned and examined using histology. In group A and B, no tissue damage was apparent under H&E staining. The positive index of PCNA was 85.3% and 84.7% for groups A and B, respectively. In group C, cellular damage was observed in the H&E stains. Necrosis, lysed cells and fragmented cellular and nuclear membranes were visible throughout the lymph nodes. The positive index of PCNA was 12.4%, significantly lower than the controls ($p<0.05$, figure 3C) indicating reduced cancer cell proliferation.

TEM imaging was performed on the lymph node sections. Group A and B appeared similar with intact cellular and nuclear membranes. In group C, significant damage to the cellular and nuclear membranes was observed with lysis occurring in some cells. Red blood cells were observed within the tumor parenchyma. The broken cellular and nuclear membranes, as well as the presence of red blood cells indicates that the vasculature was damaged, enabling the particles and blood cells to enter into the tumor parenchyma. Subsequent treatments enabled the PLGA particles to target the interstitial medium directly, exacerbating cell damage. A potential bioeffect that would decrease tumor cell proliferation is ultrasound induced cancer immunotherapy. The destruction of tumor cells in the lymph nodes may lead to immunity; the immune cells response could be amplified when exposed to the

cancer cell reminants [14]. As opposed to High Intensity Focused Ultrasound (HIFU) treatments for which cellular debris are encapsulated in the coagulated area of the lesion, it is thought that this type of cellular damage (mechanical disruption from the expanding particles) allows easier access of the immune cells to the cellular debris, thereby potentially increasing the ultrasound induced cancer immunotherapy effect.

IV. Conclusion

A new type of theranostic particle has been developed that can be used for both diagnostic and therapy purposes. The PLGA particles containing PFH can be selectively vaporized by laser irradiation within a region of interest, causing tissue damage. The anti-cancer effect could be increased by adding a chemotherapeutic drug to the core, which would be released upon vaporization. Further work is required to examine the particle kinetics, such as circulation time, particle biodistribution, and any adverse affects with administration of these agents.

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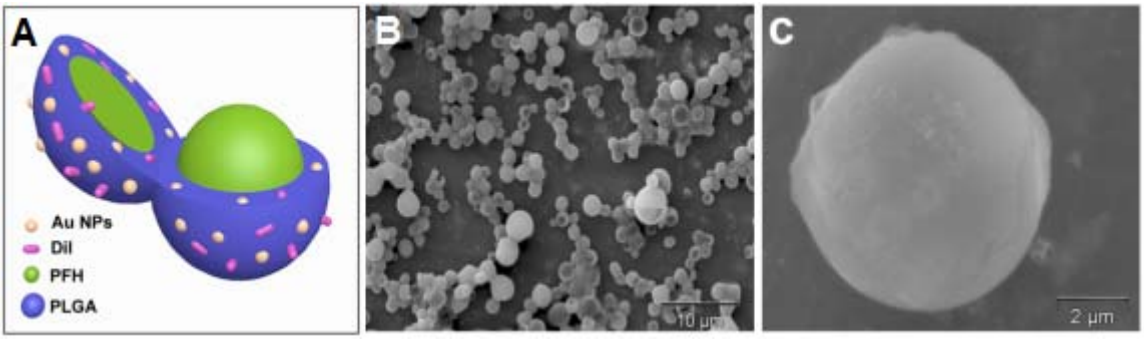

Figure 1: Morphology and structure of the PLGA particles. (A) An illustration showing the structure of PLGA particles with DiI and AuNPs in the shell, and PFH liquid in the core. (B, C) SEM images of PLGA particles at different scales. The scale bar in (B) is 10 µm, the scale bar in (C) is 2 µm.

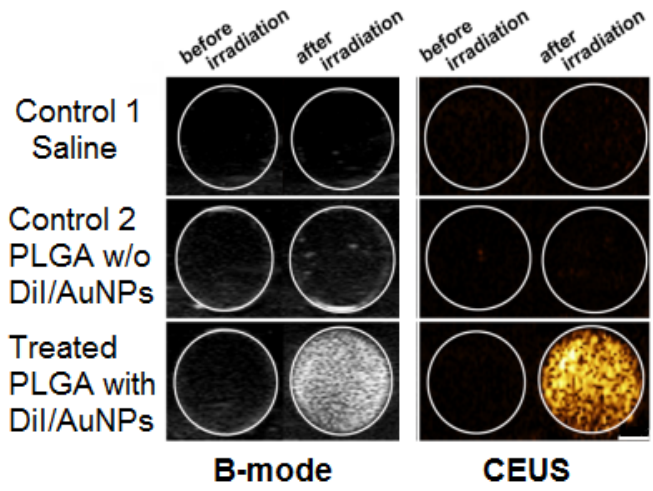

Figure 2: B-Mode and contrast enhanced ultrasound imaging (CEUS) of the PLGA particles before and after laser irradiation using a gel phantom model. Three solutions were investigated, including a saline solution only (top row), PLGA particles without DiI or AuNPs (middle row) and PLGA particles containing DiI/AuNPs (bottom row). The scale bar is 2 mm.

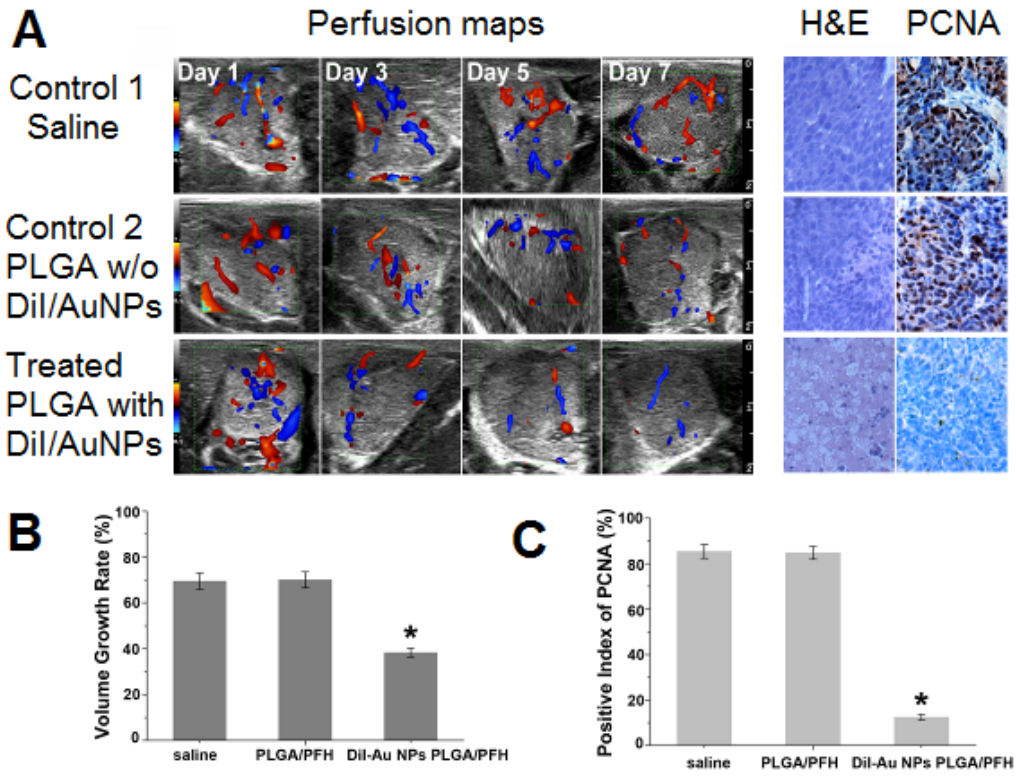

Figure 3: (A) Ultrasound images and blood perfusion maps of tumor lymph node on day 1 (before treatment), and days 3, 5 and 7 (after treatment), and images of the excised lymph nodes, H&E stains, and PCNA expression of the tumor lymph node on day 7 after therapy. (B) The volume growth rate of tumor lymph nodes in the three groups after laser irradiation (*P<0.05). (C) Positive index of PCNA in the three groups after laser irradiation (*P<0.05).