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Macrophage as cellular vehicles for delivery of nanoparticles

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Treatment of malignant brain tumors continues to challenge scientists and clinicians alike. Location of these tumors within the central nervous system (CNS), which is considered a "privileged" organ, can prevent the penetration of chemotherapeutic agents through the blood–brain barrier (BBB). To overcome this limitation, nanoparticles are taken up and transported by macrophage and then delivered directly into the CNS. In this study, we used macrophage to uptake the folate-targeted bifunctional micelles loaded with near-infrared (NIR) dye ICG-Der-01 and investigate the dynamic bio-distributions of macrophage after intravenous injection into tumor-bearing mice. *In vitro* cellular experiments by confocal microscopy indicated that the uptake of micelles in macrophage was greatly enhanced due to the folate receptor overexpression. Dynamic bio-distributions of macrophage showed a rapid clearing rate through the liver intestine pathway. In conclusion, macrophage could potentially be used as nanoparticle drug carriers and require further investigation.

Keywords: Macrophage; cellular vehicles; nanoparticles; micelles.

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1. Introduction

Malignant gliomas are the most common primary brain tumors in adults.[1] Even with substantial improvements in conventional treatments consisting of surgery, radiation therapy and chemotherapy, the prognosis for patients with this disease has not improved significantly over the past four decades: median survival is approximately 12–18 months.[1–3] One of the major factors that limits the treatment effectiveness for gliomas is the presence of the blood–brain barrier (BBB) which protects infiltrating glioma cells from the effects of anti-cancer agents. Circulating monocytes/macrophages have a natural ability to traverse the intact and compromised BBB and loaded with anti-cancer agents could be used as vectors to target tumors and surrounding tumor infiltrated tissue.[2]

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Although the macrophages plays an important physiological function in removing foreign material, cellular debris, and pathogens from circulation, these cells also play a principal role in both cancer initiation and malignant progression.[4] As tumors progress to malignancy, macrophages stimulate angiogenesis, enhance tumor cell migration and invasion, and suppress antitumor immunity. At metastatic sites, macrophages prepare the target tissue for arrival of tumor cells, and then a different subpopulation of macrophages promotes tumor cell extravasation, survival, and subsequent growth.[5,6] Macrophages accumulate and infiltrate into solid tumors, comprising up to 50% of tumor mass.[3] A number of tumor-derived chemoattractants are thought to ensure this ongoing recruitment.[7] The levels of many of these proteins in human tumors correlate positively with the numbers of macrophages present in those tumors.[4] Qian and co-workers have shown that disseminating tumor cells and host organ-derived chemokine CCL2 recruit macrophages, which facilitates efficient tumor cell metastasis seeding and growth in the distant metastatic sites.[8] A limited number of studies have investigated the ability of macrophages to deliver nanoscale drugs and imaging agents to solid tumors.[2,3,7,9–11]

Nanoparticles such as nanocapsules, nanospheres, lipsomes, micelles and others are widely applied for drug delivery.[12] We recently developed a new micelle based on poly-(NIPA-co-AAm) (PNIPAAm) achieving temporal drug delivery control, when changing the temperature of the environment slightly above the lower critical solution temperature (LCST), it will trigger a burst-like release of the encapsulated drug.[13] Folate receptor (FR) has been actively investigated for targeted delivery of therapeutics into macrophages because this receptor is selectively and highly expressed in macrophages.[14] Therefore, folate ligands as an active targeting group was incorporated to obtain improved macrophages targeting behavior.

In this study, we determined if these cells can be used as nanoparticles delivery vehicles. In this work, we used macrophage to uptake the folate-targeted bifunctional micelles loaded with near-infrared (NIR) dye ICG-Der-01[15] and investigate the dynamic biodistributions of macrophage after intravenous injection into tumor-bearing mice. Furthermore, the uptake and cell viability of macrophages were assessed.

2. Materials and Methods

2.1. *Materials*

Polyethylene glycol 400 (PEG 400), tosyl chloride (TsCl), phthalimide and hydrazine hydrate were all purchased from Huakang Technology Company (Jiangsu, China). Folic acid (folate, MW 441), N, N′-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich (Shanghai, China). N-isopropylacrylamide (NIPA, $\geq$99% in purity), Acrylamide (AAm), octadecyl acrylate (ODA), 3-mercaptopropionic acid (MPA) and azobisisobutylnitrile (AIBN) were all purchased from Aldrich-Chemie (Steinheim, Germany) and used without further purification. Hydrophobic NIR dye ICG-Der-01 was prepared in our laboratory, DMEM, fetal bovineserum (FBS), penicillin, streptomycin, trypsin-EDTA and Hochest were purchased from Gibco (Life Technologies, Shanghai, China). Triethylamine (TEA), dimethyl sulfoxide (DMSO) and other chemical solvents were all purchased from Shanghai Chemical Company. Water was double distilled.

2.2. *Synthesis of nanoparticle*

Preparation of folate modified micelles and unmodified micelles followed our previous report.[12]

2.3. *Particle size*

Particle size is increasingly recognized as an important parameter in the effective delivery of therapeutic agents by particulate vehicles, as well as size distribution can have a profound effect on the drug release profile, organ targeting and the efficiency in penetrating tissues and cells.[16] In this study, the average sizes and polydispersity of blank and ICG derivative-loaded PNIPAAm micelles were characterized by Particle Size Analyzers (Brookhaven Instruments Corporation, Austin, Texas, USA) in phosphate-buffered saline (PBS, pH 7.4).

2.4. *Cell culture*

The mouse cell lines RAW 264.7 (macrophages) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were cultured in a humidified atmosphere of 5% CO_2 at 37°C in DMEM medium supplemented with

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10% fetal bovine serum, 100 mgml^{-1} penicillin and 100 mgml^{-1} streptomycin.[17]

2.5. *Nanoparticle uptake studies*

To determine the effect of folate ligand on uptake, the visible dye Fluorescein was loaded into micelles for confocal microscopy. First, 1×10^5 RAW 264.7 cells were seeded on the confocal dish and incubated at 37°C for 24 h. Then 100 μL of two kinds of micelles were added to the culture with the cells for 1 h, respectively. At the end of the incubation period, cells were washed three times with PBS. After washing three times with PBS, the cells' nuclei were stained with Hochest (12 mgml^{-1}) for 15 min and then imaged using laser confocal microscopy.[18]

2.6. *Cell viability*

The cytotoxicity of PNIPAAm micelles was determined by employing MTT assay.[19] RAW 264.7 cells were seeded into 96-well plate and cultivated at 37°C for 24 h. After that, cells were exposed to 200 μL of PNIPAAm solutions in a series of concentrations (0.3125, 0.625, 1.25, 2.5, 5, 10 mg/mL) for another 48 h. After incubation, PNIPAAm solutions were replaced with 180 μL fresh DMEM medium and 20 μL MTT solutions (5 mg/mL). Culture medium was removed after 4 h, and 200 μL DMSO was added into each well with continuous shaking for 10 min to dissolve the formazan crystals. The absorbance at 490 nm was measured using a Microplate Reader (Model 500; BIORAD, USA). Thus the metabolic activities of the cells were defined[20] by the following equation:

$$\text{Cell viability } (\%) = \text{OD}_\text{a}/\text{OD}_\text{b} \times 100\%, \quad (1)$$

where OD_a was the optical density of the cells incubated with PNIPAAm micelles, and OD_b was the optical density of the cells treated in the absence of PNIPAAm micelles only with DMEM medium.

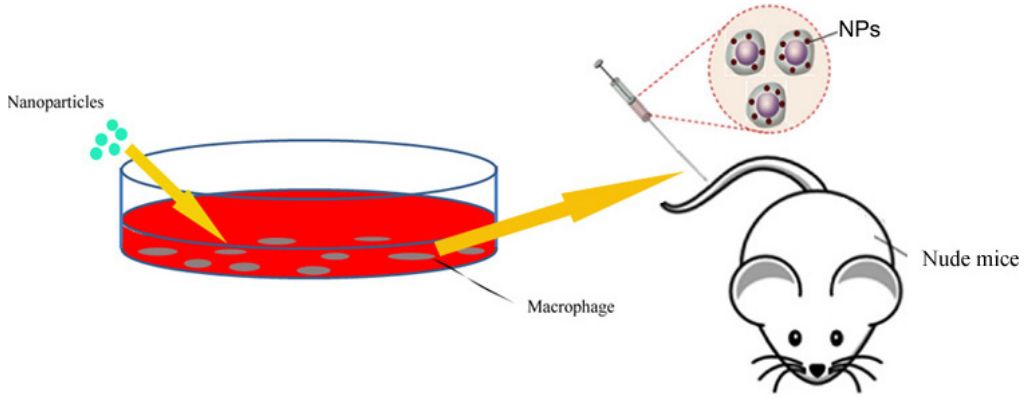
2.7. *Dynamics and bio-distribution of macrophage*

Normal nude mice were used for obtaining the dynamics of macrophages. 2×10^6 RAW 264.7 cells, loaded or not with 10 mg/mL of micelles loaded with ICG-Der-01, were intravenous injected into the subject mouse via the tail vein and monitored for 24 h. The fluorescence imaging of the pre-injection mouse was acquired and set as background imaging. All NIR fluorescent images were acquired by automatically subtracting the background imaging in the software at 1, 4, 6, 12 and 24 h from the abdomen and the back and displayed with the same fluorescence intensity scale.[21]

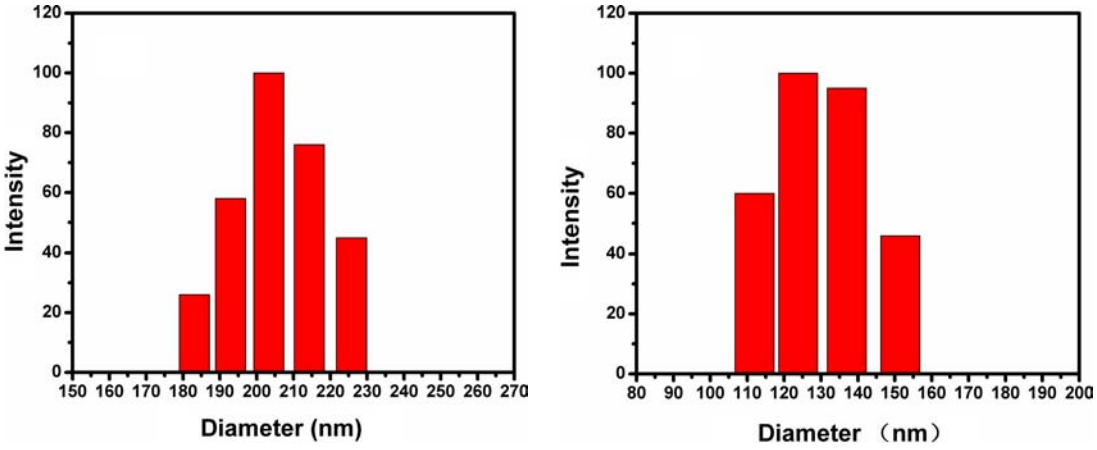
3. Results and Discussion

3.1. *Particle size*

The hydrodynamic diameters of blank micelles and ICG derivative-loaded PNIPAAm micelles in PBS determined by dynamic light scattering (DLS) were shown in Fig. 1. Protonation was avoided in PBS with pH 7.4. The size of the blank micelles was about 205 nm (Fig. 1(a)), and the size of the ICG derivative-loaded micelles was about 135 nm



Scheme 1. Schematic illustration of the cell uptake and *in vivo* experiment. The NIR dye ICG-Der-01 loaded micelles incubated with RAW 264.7 cells for 6 h. The nanoparticles loaded cells were injected into the nude mice through the tail vein.

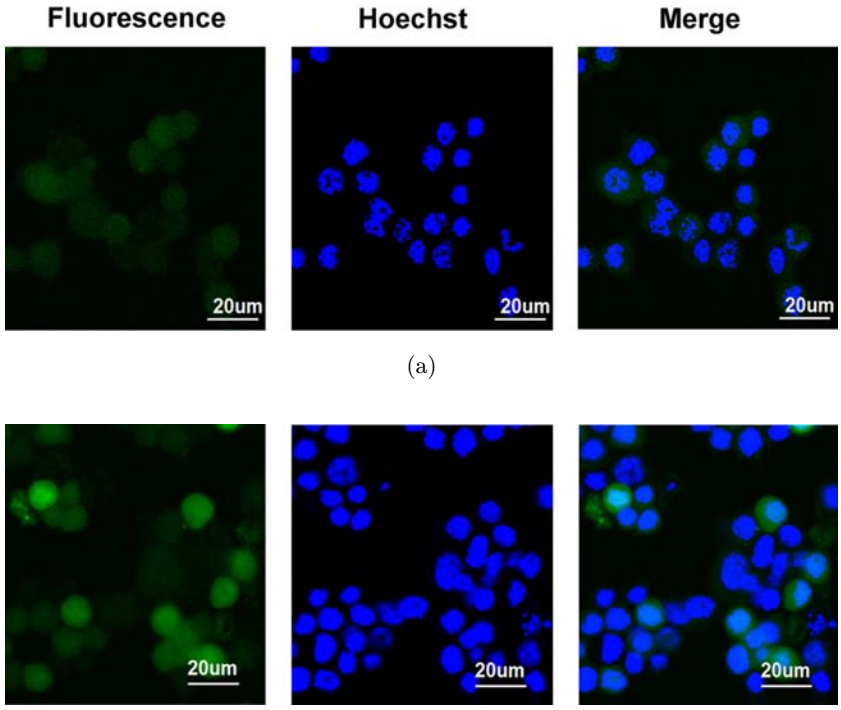


(a) (b)

Fig. 1. The hydrodynamic diameters of (a) blank micelles and (b) ICG derivative-loaded PNIPAAm micelles in PBS determined by DLS.

(Fig. 1(b)), which suggested that the micelle nanoparticles had satisfactory dimensions.

When encapsulated with hydrophobic dye ICG-Der-01, the hydrophobic interaction between ICG derivative and isopropyl would bring about much more intense hydrophobic core, representing decreased size compared to blank micelles; one ICG derivative molecule possesses two "–COO–", repulsive force increased among micelles because of the negative charge of the entirety increased after encapsulating with ICG derivative, thus acquired better dispersibility tested by Particle Size Analyzers.



(a)

(b)

Fig. 2. Representative laser confocal fluorescence microscopy images of RAW 264.7 cells incubated with (a) unmodified micelles and (b) folate modified micelles.

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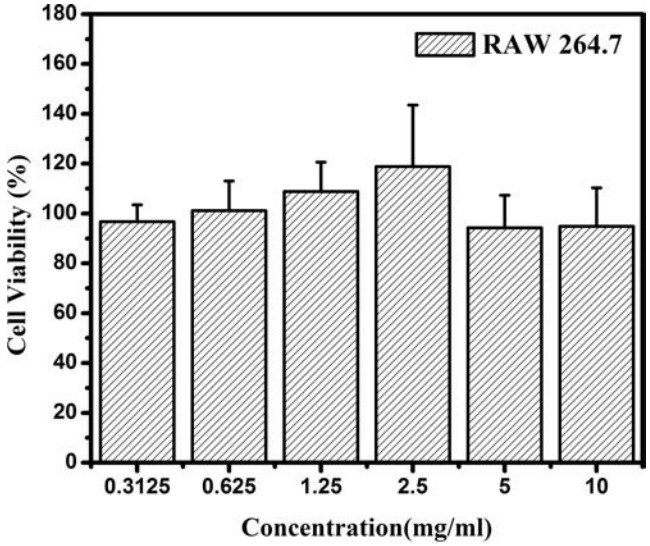

Fig. 3. Cytotoxicity study of folate modified micelles against RAW 264.7 cells.

3.2. *Cellular uptake of nanoparticles*

Scheme 1 represents the schematic illustration of the cell uptake and *in vivo* experiment. To determine the effect of folate ligand on uptake, the visible dye Fluorescein was loaded into folate modified micelles for confocal microscopy. The unmodified micelles were used as a negative control. Representative laser confocal fluorescence microscopy images of RAW 264.7 cells incubated with two kinds of micelles were shown in Fig. 2. The cells were counterstained with Hochest (blue) for the cell nucleus. The images in Fig. 2. showed that all micelles appeared to localize on the cell nucleus and fluorescence displayed in the nucleus rather than in the cytoplasm region. For the uptake ability, the cells incubated with folate modified micelles (Fig. 2(b)) gave brighter fluorescence compared with unmodified micelles incubation (Fig. 2(a)). It further proved that folate ligand greatly enhance the uptake by macrophages.

3.3. *Cytotoxicity assay of PNIPAAm micelles*

The *in vitro* cytotoxicities of folate modified PNIPAAm micelles against RAW 264.7 cells are shown in Fig. 3. The composite was proved to be weeny cytotoxicity even in 10 mg/mL by MTT assay. After the 48 h incubation, cell viabilities in the presence of micelles at different concentrations from 0.3125 to 10 mg/mL were more than 90%, even at high concentration. It was therefore presupposed

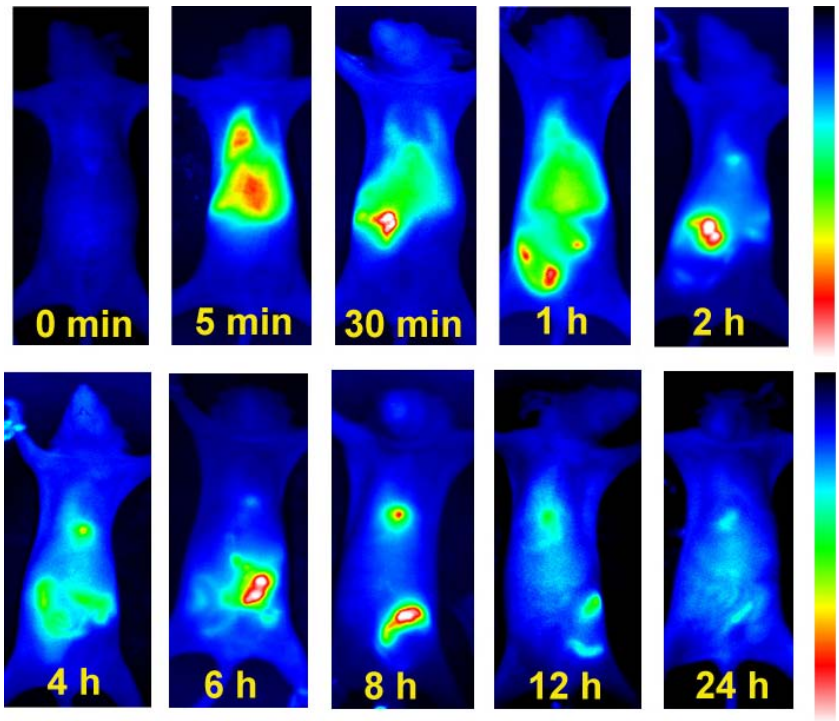

Fig. 4. NIR images of normal nude mice after intravenous injection of RAW 264.7 cells.

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that the micelles exhibited reasonable biocompatibility. The confirmation of little or nontoxicity of isopropylacrylamide-based micelles was also reported in other places.[12,22]

3.4. *Dynamics and bio-distribution of macrophage*

To better understand the physiological behavior of macrophages, the dynamics of the cells were investigated in normal nude mice prior to using the tumor-bearing mice. NIR dye ICG-Der-01 loaded micelles uptake by RAW 264.7 cells intravenously injected into the mice was monitored for 24 h, and the typical fluorescence distributions are shown in Fig. 4. As shown, the fluorescence signals of macrophages initially spread in the liver at 5 min post-injection predominantly accumulated in the liver and intestine afterward. At about 24 h post-injection, the fluorescence signal nearly disappeared in mice with residual signal in the intestine. There was no observable fluorescence signal in the bladder. The bright signal in the intestine implied that the dye and probes cleared through the liver intestine pathway. It further indicates that these hydrophilic probes have promising advantages in early tumor diagnosis.

4. Conclusion

In conclusion, we have synthesized a novel PNIPAAm micelles conjugate folate ligands which targets macrophage cells that exhibit high accumulation and infiltration into solid tumors. This study demonstrates that macrophages can serve as cellular carriers for nanoparticles. This approach could be useful to track infiltrating cells after glioma resection and for inoperable brain tumor. Due to the distribution of macrophages at the border between tumor and parenchyma, another alternative could be the encapsulation of therapeutic agents targeting the tumor microenvironment as anti-angiogenic factors.

Acknowledgments

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