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Macrophages as Cell-Based Delivery Systems for Nanoshells in Photothermal Therapy

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Abstract—Site-specific delivery of nanoparticles poses a significant challenge, especially in the brain where the blood–brain barrier prevents the entry of most therapeutic compounds including nanoparticle-based anti-cancer agents. In this context, the use of macrophages as vectors for the delivery of gold–silica nanoshells to infiltrating gliomas will be reviewed in this article. Gold–silica nanoshells are readily phagocytosed by macrophages without any apparent toxic effects, and the results of *in vitro* studies have demonstrated the migratory potential of nanoshell-loaded macrophages in human glioma spheroids. Of particular interest is the observation that, after near-infrared exposure of spheroids containing nanoshell-loaded macrophages, sufficient heat was generated to suppress spheroid growth. Collectively, these findings demonstrate the potential of macrophages as nanoshell delivery vectors for photothermal therapy of gliomas, and they certainly provide the basis for future animal studies.

Keywords—Macrophages, Nanoparticles, Gold–silica nanoshells, Gliomas, Spheroids, Blood–brain barrier.

INTRODUCTION

Metallic nanoparticles have been the subject of intense scrutiny due to their potential applications in a wide variety of diagnostic and therapeutic applications. One important criterion for the clinical use of nanoparticle-based therapy is the delivery of the nanoparticles to the target site. Cell-based vectorization of therapeutic agents has great potential for cancer therapy in that it can target and maintain an elevated concentration of therapeutic agents such as nanoparticles at the tumor site and prevent their spread into healthy tissue. The use of circulating cells such as monocytes/macrophages offers several advantages compared to mesenchymal or neural stem cells in that they are circulating cells which can be easily obtained from the patient, loaded *in vitro* with drugs or nanoparticles and reinjected into the blood stream. The use of such cells is considered to present limited risk given that macrophage accumulation occurs naturally in response to tumor development. This review will focus on the current progress in the use of gold nanoshells for photothermal therapy (PTT) of tumors with special emphasis on the use of macrophages as nanoparticle delivery platforms. The basic concept is illustrated in Fig. 1 and the experimental details, including the procedures for nanoshell loading of macrophages can be found in Baek *et al.*[2] Although much of our research has been concerned with treatment of malignant brain tumors (gliomas), the basic principles reviewed here are applicable to a wide variety of pathologies.

NANOSHELLS

Although various metallic nanoparticles have been studied, gold is particularly appealing because of its low toxicity and ease of biomolecule conjugation which enables specific targeting.[12] A variety of gold-based nanoparticles have been produced including gold nanorods, nanospheres, and nanoshells. These nanoparticles are particularly appealing for biomedical applications because of their "tunable" optical activities. Gold–silica nanoshells, consisting of a spherical

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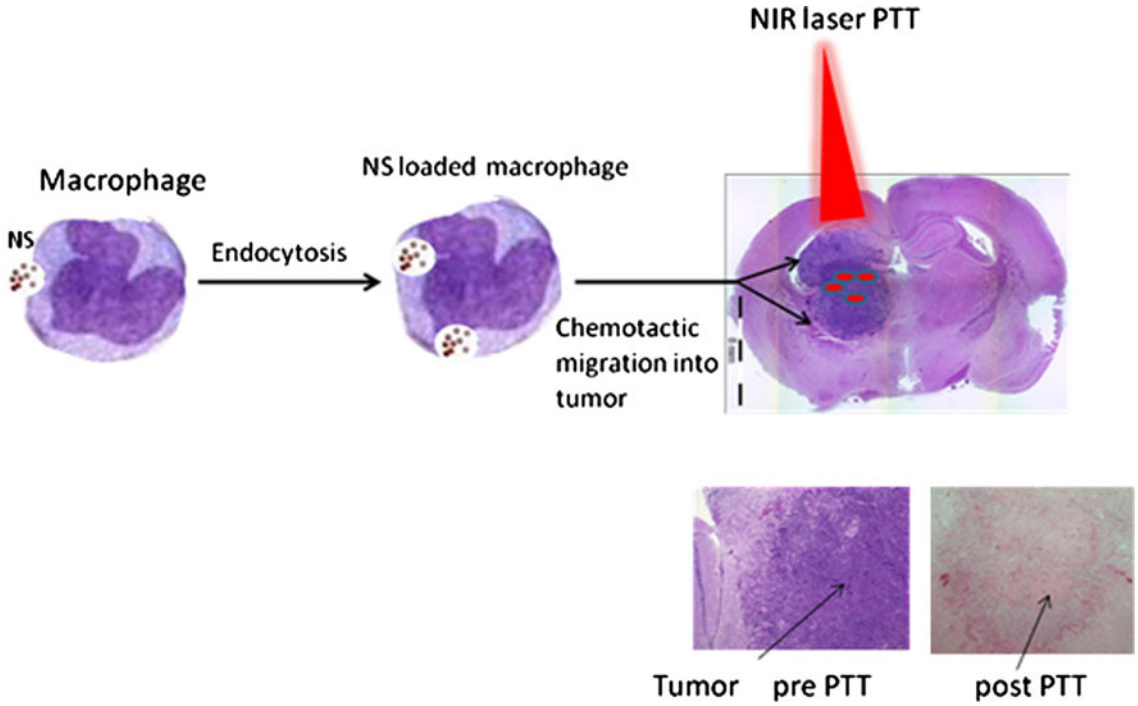

FIGURE 1. Basic concept of nanoshell-mediated PPT using macrophages as nanoshell delivery vehicles.

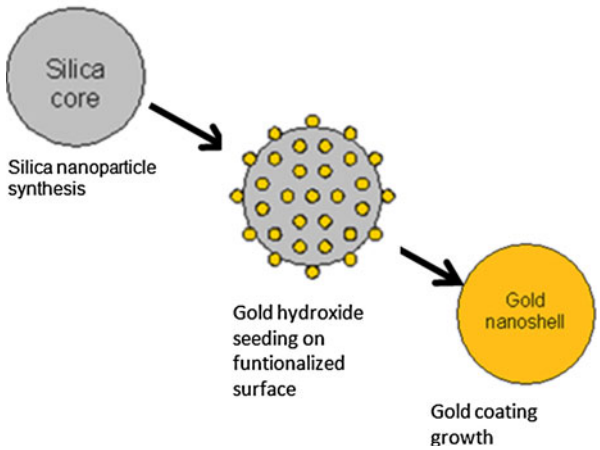

FIGURE 2. Synthesis of silica-gold nanoshells.

dielectric silica core (50–500 nm diam.) surrounded by a thin gold layer (5–20 nm), were first described by Oldenburg *et al.*[41] Synthesis of these nanoshells is relatively straightforward and has been described in a number of publications.[41,42] In brief, after nucleation and growth of the silica core and its functionalization with amine groups, colloidal gold is adsorbed onto the silica surface thereby acting as a nucleation site for further reduction of gold from $HAuCl_4$ in solution (Fig. 2). Shell thickness is controlled by the amount of $HAuCl_4$ in the solution.

The unique optical properties of metal nanoparticles are due to the interaction between light and the free conduction band electrons on the surface of the particles. When the nanoparticles (e.g., gold–silica nanoshells) are exposed to light, the electric field causes the collective oscillation of the conduction-band electrons on the surface of the particle. The coherent oscillation of the surface electrons in resonance with the electromagnetic field is termed surface plasmon resonance (SPR).[23]

Light absorption peaks at the SPR wavelength, and the absorption cross section is several orders of magnitude higher than most photoabsorbing molecules.[22] For example, the absorption cross section of gold nanoparticles is approximately six orders of magnitude higher than that of indocyanine green, a conventional organic dye, and 4–5 times that of Rhodamine 6G, the strongest absorbing organic dye.[40]

The plasmon resonance wavelength is dependent on the type of metal and its dielectric constant, as well as the shape and size of the nanoparticle.[16] Thus, another advantage over light-absorbing dyes is that metallic nanoparticles are far less susceptible to photobleaching since their absorption properties are dependent on a rigid metallic structure instead of labile molecular orbital transitions.

For gold nanoshells, the absorption resonance can be tuned from the visible to the near-infrared (NIR) simply by varying the ratio of the core-to-shell thickness. Increasing the core-to-shell ratio, by decreasing the gold shell thickness, shifts the resonance to longer wavelengths. For example, nanoshells consisting of

120 nm silica cores surrounded by thin gold shells (10–20 nm thickness) have a high absorption resonance in the NIR region of the optical spectrum (approx. 800 nm) where light has significant penetration in biological tissues.[11] This is due to a relative lack of absorption by chromophores and water in brain tissues over wavelengths ranging from 600 to 1000 nm. Since 800-nm light is efficiently converted to heat via electron–phonon interactions, these nanoshells have the potential to be used in hyperthermia applications such as PTT.

Gold nanoshells are excellent photothermal agents due to their strong absorption cross section and high optical-to-thermal energy conversion efficiency. The combined absorption cross section and photothermal conversion efficiency of gold nanoshells is approximately one million times higher than indocyanine green.[24]

The goal of PTT is to induce rapid heating in tumor tissues while minimizing thermal diffusion to surrounding tissues. The efficacy of PTT depends on a number of factors including nanoshell concentration in the target tissue, laser power density, exposure time, and blood perfusion. According to Terentyuk *et al.*,[48] the minimum temperature increase for effective PTT ranges from 10 to 20°C. A number of *in vivo* studies show that such temperature elevations can be achieved with gold nanoshell concentrations of $1–5 \times 10^9$ particles/mL, laser power densities of 1–5 W cm^{-2}, and treatment exposures of 1–5 min.[48] It should be noted that, failure to achieve adequate temperature elevations, may result in adverse effects because of the production of heat shock proteins which confer resistance to tumor cells.[9]

Hirsch *et al.*[19] were the first to demonstrate PTT using gold–silica nanoshells. There have been a number of *in vitro* studies evaluating the potential of these nanoshells in a variety of cancer cell lines including human breast,[31–33] prostate,[14,47] brain[5] and liver.[30] Nanoshell-mediated PTT has shown to be effective against xenografted subcutaneous tumors in mice and allografted tumors in dogs.[11,40,46] Nevertheless, the larger size of gold nanoshells, compared to other gold nanostructures, poses a significant challenge for some applications including the treatment of malignant gliomas where the blood–brain barrier (BBB) prevents the entry of most therapeutic agents into the brain.

BIODISTRIBUTION OF GOLD–SILICA NANOSHELLS

Systemic administration of nanoshells is required in the vast majority of clinical applications. After administration, nanoshells passively localize in tumors via the enhanced permeability and retention (EPR) effect by passing through fenestrations in the angiogenic tumor vasculature.[36] The nanoshells are preferentially retained in the tumor because of impaired lymphatic drainage associated with tumor development.

The biodistribution of nanoshells is affected by a number of factors including their size and surface charge. Owing to their negatively charged surface, serum proteins tend to bind to nanoshells during circulation resulting in their rapid uptake and removal by cells of the reticuloendothelial system (RES).[8,17] A commonly used strategy to increase circulation time is to coat nanoshells with chains of polyethylene glycol (PEG) which increases particle size thus preventing filtration by excretory organs. Pegylation also prevents protein binding to the particle surface and hence delays recognition by cells of the RES.

The biodistribution of pegylated gold–silica nanoshells has been studied in subcutaneous murine tumor models using neutron activation analysis[27] and inductively coupled plasma-mass spectrometry[11] to assay for gold. Peak tumor accumulation of nanoshells was observed approximately 24 h after intravenous administration. The highest concentration was observed in the liver and spleen. In the study reported by James *et al.*,[27] the concentration of gold in the liver and spleen at 24 h was 20–25× the gold concentration observed in the tumor, suggesting that $<5\%$ of the injected dose was delivered to the tumor. Data from both studies show that only trace amounts of gold were found in normal brain suggesting that the BBB is an effective barrier against the passage of nanoshells into the brain.

MALIGNANT GLIOMAS

Primary intracranial neoplasms account for 1.5% of all cancer deaths.[1] Approximately half of these are glioblastoma multiforme (GBM)—the most aggressive type of glioma. In a study published in 1932, Cushing noted that the mean survival of GBM patients ranged from 6 to 9 months.[10] Even with the best available treatments consisting of surgery, chemotherapy, and radiation, median survival at present is approximately 14 months.[37] Thus, despite impressive advancements in neuroimaging and neurosurgery, and the development of a wide variety of anti-cancer agents, there has been relatively little improvement in the survival of GBM patients over the past eight decades.

The poor prognosis of GBM patients is due primarily to the aggressive and infiltrating nature of these tumors: in most cases, glioma cells have already infiltrated 2–3 cm into normal brain at the time of bulk tumor resection.[50] These infiltrative tumor cells are

protected by the BBB, formed by tightly connected brain capillary endothelial cells, which normally prevent harmful substances from entering the brain.[3,25] Unfortunately, few anti-cancer agents can effectively cross this barrier to target infiltrating glioma cells.[34] Failure to eradicate these cells inevitably results in tumor recurrence and further treatments are usually palliative in scope. Therefore, destruction of infiltrating tumor cells is a critical step for curing malignant gliomas such as GBM. This cannot be accomplished until methods are developed to: (1) deliver drugs or carriers across the BBB at selected sites, or (2) selectively disrupt this protective barrier.

MACROPHAGES AS VECTORS FOR THE DELIVERY OF NANOSHELLS

Macrophages are a class of white blood cells derived from myeloid progenitor cells in the bone marrow. As their name implies (big eaters, from *makros* "large" + *phagein* "eat"), the primary functions of macrophages are to engulf and digest material foreign to the body. Their precursors are monocytes that differentiate into macrophages after their migration from the circulation into tissues.[44] For example, microglia are the resident tissue macrophages of the central nervous system.[6] Macrophages are the first to mediate host immune responses against foreign objects. In addition to their role as eliminators of invading pathogens, macrophages can also release growth factors, cytokines, interleukins, and nitric oxide that trigger other inflammatory responses and mediate repair of damaged tissues.[21,38,45]

Circulating monocytes/macrophages have a natural ability to traverse the intact and compromised BBB and undergo differentiation into long-lived brain-resident macrophages and microglia.[18] Macrophage migration into the CNS is a highly regulated function and is dependent on cell–cell interactions involving tumor cells, endothelial cells and astrocytes. Furthermore, the local release of factors that promote BBB permeability also play a role. Tumor-associated macrophages (TAMs) are frequently found in and around GBMs in both experimental animals and patient biopsies. In GBM, it has been shown that TAMs can constitute up to a third of the tumor mass.[13] The use of macrophages as a cell-based delivery vehicle for nanoparticles stemmed from the observation that the systemic administration of paramagnetic nanoparticles for MRI resulted in their ingestion by endogenous macrophages that subsequently migrated and accumulated in and around tumors.[28,38,43] This was also the case when exogenous *in-vitro*-labeled macrophages were injected intravenously.[49] Collectively, these observations indicate a local synthesis of chemo attractive factors in gliomas and that inflammatory cells can pass through the blood–brain tumor barrier (BBTB) as well as the brain adjacent-to-tumor (BAT) region where the concentration of infiltrating glioma cells is the highest. Macrophages loaded with drugs, nanoparticles, or photosensitizers could therefore be used to target tumors and surrounding tumor-infiltrated tissue.[7,25,44,49,51]

The ability of macrophages to phagocytose several types of nanoparticles including gold nanoshells, the first step in the cell vectorization of nanoshells, has previously been investigated.[7,28] From studies conducted in the authors' lab, two-photon microscopy images of murine macrophages incubated for 24 h with nanoshells revealed the presence of nanoparticles dispersed throughout the cellular cytoplasm (Fig. 3) In addition, aggregates of nanoshells in vacuoles within the cytoplasm of macrophages have been visualized with transmission electron microscopy.[4] The effects of aggregation on the plasmonic properties of the nanoshells are uncertain; however, based on preliminary visible and infrared absorption studies conducted in our lab, there does not appear to be a significant shift in the absorption peak of single nanospheres in solution compared to aggregated nanospheres in macrophates.

When nanoparticles are injected into the circulation, they are quickly engulfed by fixed (Kupffer) cells, spleen, and bone marrow as well as circulating monocytes/macrophages and are rapidly removed from the circulation. Various strategies have been developed to prolong their systemic blood circulation time allowing more of these particles to be delivered to the tumor site. Among the various polymers used for this purpose, PEG is currently the most popular and the most effective in prolonging circulation time. On the other hand, the strategy of employing *in vitro* loading of macrophages vectors would dictate a maximum uptake of nanoparticles by the macrophages. Although the % uptake of bare nanoshells compared to PEGylated ones is considerably greater, sufficient numbers are nevertheless taken up to provide efficient PTT.[47] Not surprisingly, owing to the biocompatibility of gold, the nanoshells were shown to be nontoxic to murine macrophages.

MACROPHAGE MIGRATION IN HUMAN GLIOMA SPHEROIDS

The migratory potential of nanoshell-loaded macrophages has been investigated in human glioma spheroids. This simple *in vitro* model was chosen, since it eliminates many of the problems intrinsic to monolayers

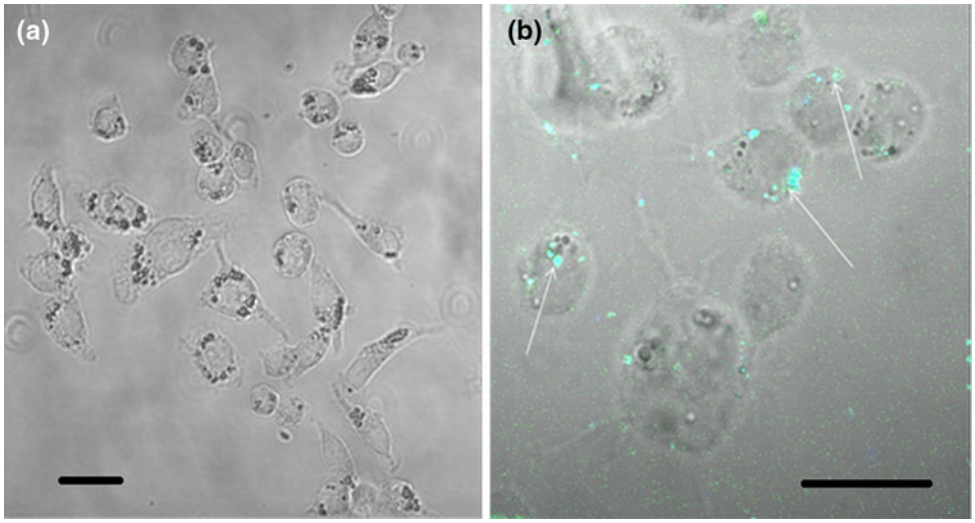

FIGURE 3. Two-photon fluorescence micrograph of (a) empty or (b) silica–gold nanoshell-loaded murine macrophages. The scale bar in each image denotes 10 μm. Nanoshell aggregates inside macrophages are shown by green reflectance (white arrows).

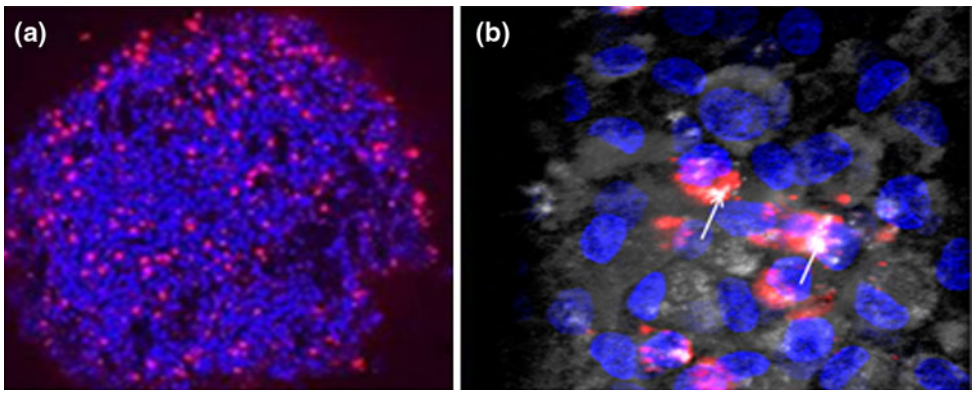

FIGURE 4. Two-photon fluorescence image of (a) nanoshell-loaded macrophage-infiltrated spheroid (low magnification—400 × 400 μm); (b) nanoshell-loaded macrophage migration into human glioma spheroids (high magnification—20 × 20 μm). The cell nucleus was stained with Hoechst 33342 (blue), while the macrophage cytoplasm was stained with PKH26 Red Fluorescent (red). Nanoshell aggregates inside macrophages are shown by white reflectance (white arrows). Images were acquired at a depth of approximately 80 μm.

and animal models. Multicell spheroids are three-dimensional aggregates of tumor cells that mimic tumor nodules prior to their vascularization. Tumor cells within spheroids show a higher degree of morphological and functional differentiation than cells grown in monolayer culture. They also display growth kinetics, metabolic rates, and resistance to radiotherapy, and photodynamic and chemotherapy similar to tumor cells *in vivo* (for a review, see Madsen *et al.*[35]). The primary drawback of spheroids is their inability to model the tumor vasculature which is important in thermal therapies since blood serves as an important thermoregulatory mechanism. Nevertheless, spheroids represent a more realistic tumor model than monolayers and, as such, can provide better guidance regarding the choice of laser parameters to be used in animal studies.

Two-photon fluorescence microscopy studies have confirmed the migratory potential of macrophages co-incubated with human glioma spheroids (Fig. 4).[2] The number of macrophages infiltrating the spheroids increased over the first 12 h of co-incubation and then appeared to approach saturation levels (Fig. 5). The slight differences in migration kinetics observed between empty and nanoshell-loaded macrophages suggest that nanoshells do not significantly affect the migratory potential of macrophages. The uniform distribution of macrophages observed in this study is in contrast with previous studies which have demonstrated macrophage accumulation in the inner hypoxic rim around the necrotic core of the spheroid.[7,44] This discrepancy is likely due to differences in spheroid-formation techniques. For example, the centrifugation technique used by Baek *et al.*[2] produced relatively few

necrotic cells in the core region of the spheroid during the first 48 h, when the majority of co-incubation studies were performed.

Macrophage accumulation around the necrotic core of spheroids is consistent with *in vivo* findings that show high concentrations of TAMs in hypoxic tumor areas where it is hypothesized that, being phagocyte scavenger cells, they are attracted by signals released by cells undergoing necrosis.[29,39] In addition, it has been shown that hypoxic tumor cells recruit TAMs by an increased rate of production of macrophage chemo attractants such as vascular endothelial growth factor (VEGF). After migration, hypoxic tumor cells entrap TAMs by down-regulating chemokine receptors and chemo attractants and up-regulating macrophage migration inhibitory factor. This phenomenon is also likely in GBM, however, since the vast majority of the necrotic core found in these tumors is removed during cytoreductive surgery, it is the TAMs that have infiltrated the BAT region which are of therapeutic interest.

The chemotaxic effects of glioma spheroids on macrophages have been investigated in a collagen gel matrix model consisting of human glioma spheroids implanted in dried rat tail type I collagen.[20] As illustrated in Fig. 6, macrophage migration toward spheroids was observed, as was penetration into the spheroids.

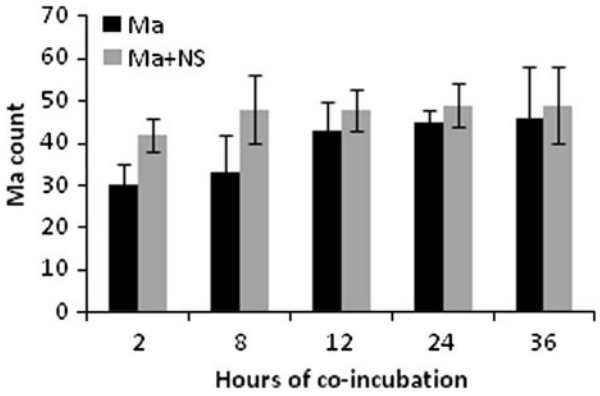

FIGURE 5. Infiltration of empty and nanoshell (NS)-loaded macrophages (Ma) into glioma spheroids with increasing co-incubation times. 2 × 10^4 macrophages were incubated for 2, 8, 12, 24, or 36 h with 700-μm-diameter spheroids. Macrophage counts were obtained from 15-μm-thick two-photon microsections located 100 μm into the spheroid. The values shown represent the average macrophage counts of 4 spheroids ± SD.

PHOTOTHERMAL THERAPY OF HUMAN GLIOMA SPHEROIDS

In vitro studies using human glioma spheroids have demonstrated the efficacy of nanoshell-mediated PTT.[2] For example, two-photon micrographs of PTT-treated spheroids, consisting of human glioma cells and nanoshell-loaded macrophages, show significant cell death after exposure to NIR laser light (Fig. 7). Not surprisingly, increased laser irradiances resulted in decreased spheroid growth (Fig. 8)—complete suppression of spheroid growth was observed at irradiances of 14 W cm^{-2}, while the thermal energy produced after exposure to 28 W cm^{-2} for a few seconds resulted in explosive dissociation of the spheroids. The dependence of the number of loaded macrophages on PTT efficacy has been investigated in hybrid spheroids produced from a suspension mixture of tumor cells and nanoshell-loaded macrophages by the centrifugation method of Ivascu and Kubbies.[26]

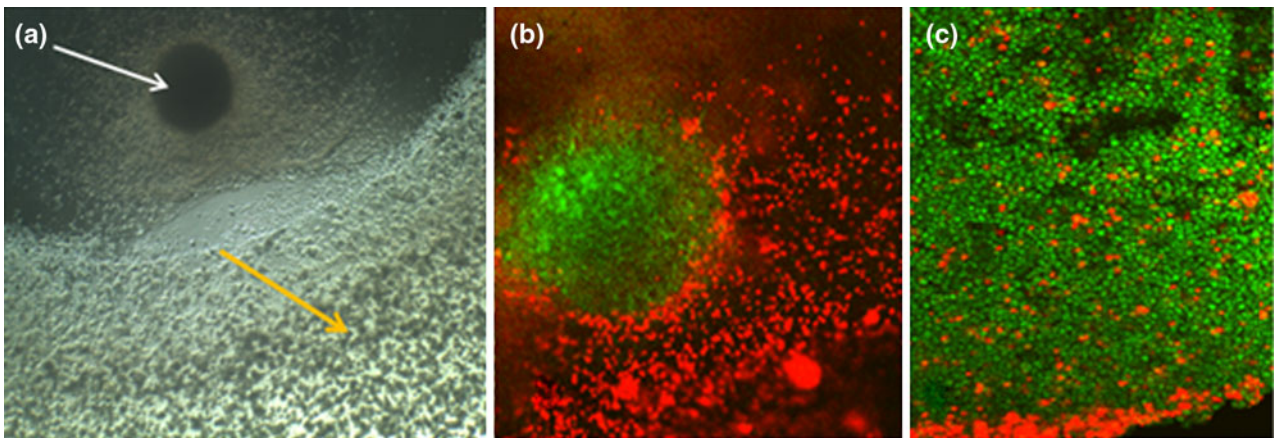

FIGURE 6. (a) Light microscope image of collagen migration assay consisting of two collagen layers, one containing a single tumor spheroid (white arrow) and the other containing distributed macrophages (yellow arrow). (b) Two-photon fluorescence image demonstrating macrophage (red cells) migration from the macrophage collagen layer (a) toward the spheroid consisting of glioma cells transfected with green fluorescent protein (GFP). (c) High-magnification tomographic image demonstrating penetration of macrophages (red) into the GFP spheroid (green).

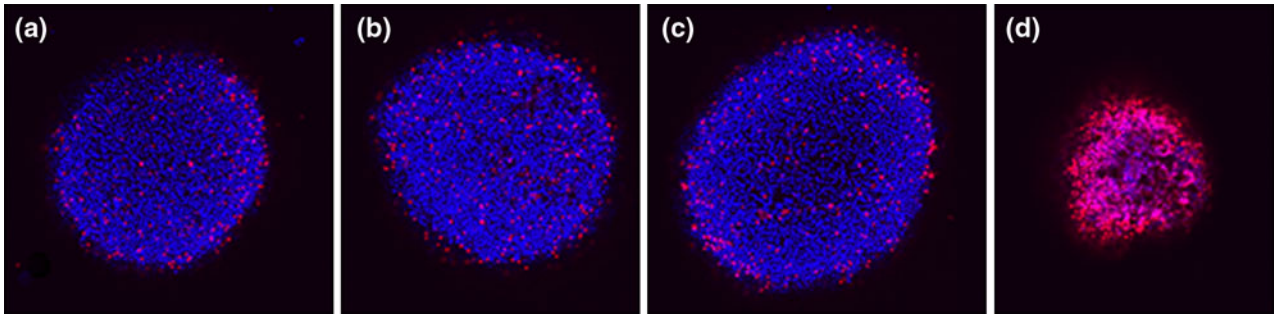

FIGURE 7. Live/dead assay of PTT-treated spheroids. Two-photon micrographs were made at planes 100 μm into the spheroids with simultaneous excitation of the two dyes (blue—live; red—dead). (a) Spheroid control (no treatment); (b) Spheroid + nanoshell-loaded macrophages; (c) Spheroid + empty macrophages + PTT (dark control); and (d) Spheroid + nanoshell-loaded macrophages + PTT. PTT-treated spheroids were irradiated with 800-nm laser light for 10 min at an irradiance of 14 W cm^{-2}. Images in a-c were acquired 12 days post PTT, while the image in d was acquired 2 days post PTT. In all cases, a 600 × 600-μm field-of-view was used.

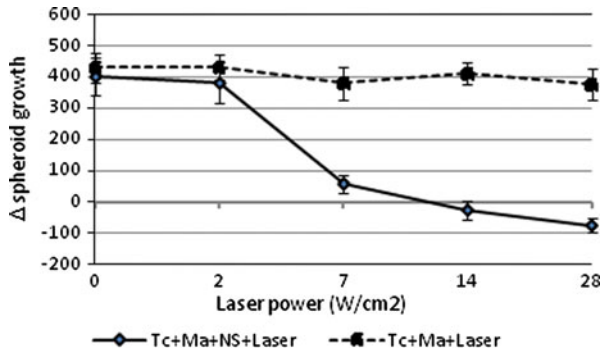

FIGURE 8. Effects of laser irradiance on spheroid growth. Preformed spheroids incubated with either empty (Tc + Ma) or nanoshell-loaded macrophages (Tc + Ma + NS) for 24 h, transferred and irradiated with increasing irradiances for 10 min. The values shown represent the average diameter after 14 days of incubation minus the initial diameter of the spheroid (Δ spheroid growth).

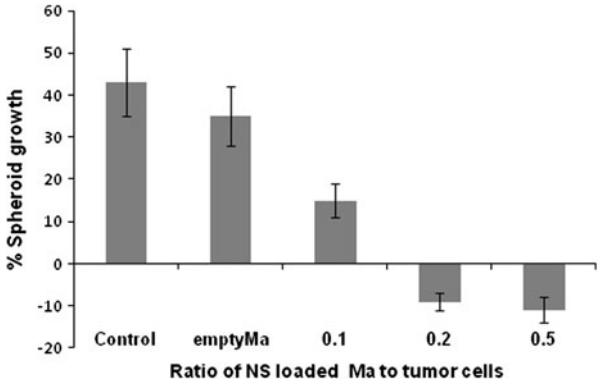

FIGURE 9. Effects of increasing numbers of nanoshell-loaded macrophages on spheroid growth after PTT. Spheroids were irradiated with 800-nm laser light for 10 min at an irradiance of 14 W cm^{-2}. The values shown represent the average spheroid diameter 14 days post PTT as a % increase of the initial size. The results show the average of eight spheroids per group (±SE).

This technique produces spheroids of known macrophage-to-tumor cell ratios with macrophages distributed uniformly throughout the spheroid. As illustrated in Fig. 9, significant growth suppression was observed in hybrid spheroids consisting of 10% macrophages. Complete growth cessation was observed at a concentration of 20% loaded macrophages.

SUMMARY

Macrophages have the potential to increase the delivery of nanoparticles, such as gold–silica nanoshells, to a wide variety of tumors including GBM. As evidenced from biodistribution studies, nanoshells do not cross the patent BBB. In contrast, macrophages readily traverse the BBB where residual glioma cells are found after surgical resection. Nanoparticles tend to accumulate in tumors passively via the EPR effect. Although nanoparticles can penetrate the leaky vasculature in tumors, the extracellular matrix of solid tumors presents a formidable transport barrier that limits penetration thus limiting the efficacy of nanoparticle-mediated therapies.[15] In contrast, cells are capable of migrating through the extracellular matrix, especially inflammatory cells such as macrophages. Systemically injected macrophages will either track to the tumor site or be eliminated in the RES. Methods which would increase the ability of exogenous-loaded macrophages to migrate to the BAT, making them available for postoperative therapeutic modalities are vital to the possible implementation of this technique for future patient treatments.

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