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Multifunctional Particles for Melanoma-Targeted Drug Delivery

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Abstract

New magnetic-based core-shell particles (MBCSP) were developed to target skin cancer cells while delivering chemotherapeutic drugs in a controlled fashion. MBCSP consist of a thermo-responsive shell of poly(*N*-isopropylacrylamide-acrylamide-allylamine) and a core of poly(lactic-co-glycolic acid) (PLGA) embedded with magnetite nanoparticles. To target melanoma cancer cells, MBCSP were conjugated with Gly-Arg-Gly-Asp-Ser (GRGDS) peptides that specifically bind to the $\alpha_5\beta_3^+$ receptor of melanoma cell. MBCSP consist of unique multifunctional and controlled drug delivery characteristics. Specially, they can provide dual drug release mechanisms (a sustained release of drugs through degradation of PLGA core and a controlled release in response to changes in temperature via thermo-responsive polymer shell), and dual targeting mechanisms (magnetic localization and receptor-mediated targeting). Results from *in vitro* studies indicate that GRGDS-conjugated MBCSP has an average diameter of 296 nm and exhibit no cytotoxicity towards human dermal fibroblasts up to 500 $\mu\text{g ml}^{-1}$. Further, a sustained release of curcumin from the core and a temperature-dependent release of doxorubicin from the shell of MBCSP were observed. The particles also produced a dark contrast signal in magnetic resonance imaging. Finally, the particles were accumulated at the tumor site in a B16F10 melanoma orthotopic mouse model, especially in presence of a magnet. Results indicate great potential of MBCSP as a platform technology to target, treat, and monitor melanoma for targeted drug delivery to reduce side effects of chemotherapeutic reagents.

Keywords

Biodegradable polymers; Magnetite nanoparticles; Thermo-responsive polymers; Targeted drug delivery; Melanoma

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1. Introduction

Current treatment methods for melanoma, the most prevalent form of skin cancer, have several limitations such as low target specificity, severe side effects, and drug resistance [1]. Metastatic melanoma cells may invade other organs, leading to a hindered response to chemotherapeutic drugs and a decrease in patient survival rate [2]. To address some of these issues, substantial progress has been made in the development of particulate drug delivery systems. It is well established that targeted drug delivery by nanoparticles is more effective and less harmful than the conventional systematic delivery of chemotherapeutic drugs [3]. Nanoparticles are thought to be ideal carriers to efficiently deliver the drugs directly to the tumor site, thereby reducing systemic toxicity and side effects [4]. Different types of functional nanoparticles have been designed to target or accumulate in cancer tissues via either receptor-mediated targeting or passive targeting, respectively [5], leading to an increase in therapeutic efficiency while minimizing side effects.

Conjugation of targeting moieties like antibodies, peptides, or aptamers to the particles has been used to allow for active targeting of cancer cells and overcome the limitations of passive targeting [6]. For an instance, Arg-Gly-Asp (RGD)-functionalized nanoparticles have been used for both targeted drug delivery and imaging applications, especially in melanoma skin cancer [7, 8]. Furthermore, Gly-Arg-Gly-Asp-Ser (GRGDS) peptides have been shown to increase the specific binding of the particles to the $\alpha_5\beta_3^+$ melanoma cells [9]. Many recent works have also emphasized the development of a multifunctional cancer therapeutic system with both diagnosis and treatment capabilities by incorporating various polymers, organics, and inorganic materials [10].

Several particle devices developed for melanoma cancer therapies have demonstrated varying degrees of success. These particles include hydrophobically modified glycol-chitosan nanoparticles for Paclitaxel-based chemotherapy [11], magnetic-based cationic liposomes for a combination of hyperthermia and chemotherapy [12], and Realgar nanoparticles for transdermal drug delivery [13]. Recently, several studies have demonstrated the effectiveness and advantage of combinatorial treatments of cancer by utilizing multiple drugs due to their additive or synergistic effects on overcoming cancer-drug resistance [14]. However, combined therapies with two or more drugs often require multiple administrations on the same or different schedule (long or short term regimes), making this combinatorial treatment inconvenient for systematic administration. Drug-loaded particles have been developed to overcome these limitations by providing a sequential drug release via either multi-layer or single-layer particles. Multi-layer particles provide multiple compartments for drug loading, whereas single-layer particles can be loaded with either multiple drugs or a combinatorial drug conjugated to hydrolysable linkers [14, 15]. Despite such advances, these particles lack the ability of combined therapies, as well as simultaneous targeted treatment and monitoring while providing efficient drug release.

To provide combined therapy, controlled release of drugs in response to changes in temperature and hyperthermia, with monitoring the disease and treatment progress, we have previously developed poly(*N*-isopropylacrylamide-acrylamide-allylamine) (PNIPAAm-AAm-AH)-coated magnetic particles for controlled drug delivery in response to changes in temperature [16]. At temperatures below lower critical solution temperature (LCST, 39–40°C), PNIPAAm-AAm-AH is hydrophilic and swells. At temperatures above LCST, the polymer becomes hydrophobic, shrinks, and releases drugs. However, only hydrophilic drugs could be loaded in and released from these particles, due to the temperature-sensitive properties of PNIPAAm-AAm-AH. We have also developed multi-layer particles by incorporating PNIPAAm-AAm-AH-coated magnetic particles in a core of poly(lactic-co-glycolic acid) (PLGA) shell [17]. These particles could be loaded with both hydrophilic and

hydrophobic drugs and provide both sustained and burst releases of drugs. However, not all of the PNIPAAm-AAm-AH-coated magnetic particles were in the core of PLGA; some were present on the surface of PLGA. Moreover, several PNIPAAm-AAm-AH-coated magnetic particles were incorporated into the core, which released drugs in response to changes in temperature. However, the released drugs were still trapped in the PLGA shell, diminishing the advantage of temperature-sensitive drug release.

To provide targeted and combined therapies and overcome the limitations of our previous drug delivery systems, we have now developed new magnetic-based core-shell particle (MBCSP) drug delivery system. The particle core is comprised of PLGA-magnetite to load either hydrophilic or hydrophobic anticancer drugs and provide sustained release of drugs via the polymer degradation. In addition, the shell is comprised of thermo-responsive polymer (PNIPAAm-AAm-AH) for temperature-dependent release of anti-cancer drugs. The presence of magnetite (iron oxide nanoparticles) in the MBCSP permits the accumulation of particles at the target site by the application of a magnetic field [18]. Application of an AC magnetic field causes vibrations and heat generation in magnetite, which is useful for hyperthermia treatment of cancers. Thus, MBCSP would provide combined therapies to kill cancer cells by both hyperthermia and drug release from a thermo-responsive polymer shell in response to changes in temperature. Simultaneous magnetic resonance imaging (MRI) and hyperthermia treatment of tumors would also be possible due to the inclusion of magnetite into the MBCSP [19]. Importantly, the application of magnetite for targeting, hyperthermia, and imaging of melanoma is useful and easier, due to easily accessible superficial locations of melanoma cancers. Furthermore, the aggregation and toxicity of bare magnetite could be reduced significantly, due to the polymeric shell on the magnetite [20].

2. Materials and methods

2.1. Materials

PLGA (50:50, Lakeshore Biomaterials, Birmingham, AL), magnetite (Meliorum technologies, Rochester, NY), doxorubicin (Tocris Bioscience, Ellisville, MO), and polyvinyl alcohol (PVA), curcumin, *N*-isopropylacrylamide (NIPAAm), sodium dodecyl sulfate (SDS), *N,N'*-methylenebisacrylamide (BIS), potassium persulfate (KPS), vinyltrimethoxysilane (VTMS), ethanol, acetic acid, *N,N,N',N'*-tetramethylethylenediamine (TEMED), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimidehydrochloride (EDC), acrylamide (AAm), allylamine (AH), and dichloromethane (DCM) were purchased from Sigma-Aldrich (St. Louis, MO). B16F10 mouse melanoma cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). Dulbecco's modified eagle's medium (DMEM), neonatal calf serum, penicillin-streptomycin, and trypsin were purchased from Invitrogen (Carlsbad, CA). GRGDS peptides were purchased from Anaspec (Fremont, CA).

2.2. Synthesis of MBCSP

The MBCSP were synthesized via a step-by-step synthesis procedure. Firstly, PLGA nanoparticles were synthesized by oil-in-water emulsion technique and loaded with either bare magnetite or VTMS-functionalized magnetite as described previously [21]. In brief, magnetite nanoparticles were added to PLGA solution (100 mg in 5 ml DCM), which was then emulsified in PVA (2% w/v) solution to form PLGA-magnetite particles. The PLGA-magnetite core was further incorporated with PNIPAAm-AAm-AH polymer shell by free radical polymerization [16]. Briefly, carboxyl function groups of PLGA-magnetite particles (20 mg) were activated via EDC and NHS (1:1); [22] and monomers of NIPAAm, AAm, and AH were copolymerized on the surface of these particles by a free radical

polymerization to form MBCSP [16]. MBCSP were further conjugated with GRGDS peptides via carbodiimide chemistry as previously described [22].

2.3. Characterization of MBCSP

The iron content of MBCSP prepared using either bare magnetite or VTMS-functionalized magnetite was evaluated by an iron assay as described earlier [16]. Transmission electron microscopy (TEM, Technai, JEOL 1200 EX, Tokyo, Japan) and dynamic light scattering (DLS, ZetaPals, Brookhaven Instrument, Holtsville, NY) were used to assess particle morphology, size, and surface charge. In addition, after each step of the particle synthesis, Fourier transform infrared spectroscopy (FTIR, Nicolet 6700 FT-IR spectrometer, Thermo Fisher Scientific, Madison, WI) was performed to confirm the successful layer-by-layer synthesis. To determine magnetic properties of bare magnetite and MBCSP, superconducting quantum interference device (SQUID, Quantum design) magnetometer was used. Particle samples consisting of the same amount of magnetite were embedded in epoxy resin capsules and hysteresis loops were obtained by varying the magnetic fields at the room temperature.

2.4. Drug loading and release from MBCSP

For drug loading and release studies, hydrophobic curcumin and hydrophilic doxorubicin hydrochloride drugs were used. Curcumin, a polyphenolic compound, was chosen as a cancer drug model, since it is known to possess anti-angiogenic properties and inhibitory effects on organ metastasis caused by melanoma [23]. Curcumin (30 mg) was loaded into the core of the MBCSP along with VTMS-functionalized magnetite during the emulsion process. The curcumin loading efficiency was calculated by an indirect method using the following formula.

$$\text{Percentage loading efficiency} = \frac{\text{amount of drugs used} - \text{amount of drugs in supernatant}}{\text{amount of drugs used}} \times 100\%$$

For curcumin release studies, the curcumin-loaded MBCSP (1 mg ml^{-1}) were suspended in PBS and incubated at 25°C (below LCST of thermo-sensitive shell) and 41°C (above LCST of thermo-sensitive shell) to analyze the effect of temperature on the curcumin release from the core. At each predetermined time point up to 25 days, the drug release samples were collected for analysis using UV-Vis spectrometer (Infinite M200, Tecan, Durham, NC) at a wavelength of 428 nm. Similar methods were followed to determine the loading efficiency and release kinetics of doxorubicin. Doxorubicin, a potent anticancer agent against several forms of cancers, like thyroid, breast, stomach, and lung cancers, has also exhibited significant anticancer effects against growth and metastasis of melanoma [10], making it a suitable choice for our drug delivery system. Freeze-dried MBCSP (10 mg) were suspended in doxorubicin (0.02% w/v in PBS) solution and incubated at 4°C for 3 days, while gently stirring to allow loading of the drug into the thermo-sensitive polymer shell. After 3 days, the drug-loaded MBCSP were collected using a magnet and the supernatant containing the unloaded drug was used for determination of the doxorubicin loading efficiency indirectly. At each predetermined time point up to 25 days, the drug release samples were collected and analyzed at an excitation wavelength of 470 nm and an emission wavelength of 585 nm using the UV-Vis spectrometer (Infinite M200, Tecan, Durham, NC).

2.5. Cell culture and cytotoxicity of MBCSP

Human dermal fibroblasts (HDFs, origin: human skin epidermis, obtained from Invitrogen), B16F10 melanoma cells (origin: murine skin, purchased from ATCC), and NIH/3T3

fibroblasts (origin: murine embryo, purchased from ATCC) were cultured in complete DMEM supplemented with 10% serum and 1% penicillin-streptomycin. Cultures were maintained at 37°C and 5% CO₂ in a humid atmosphere. The toxicity of MBCSP and GRGDS-MBCSP on HDFs was evaluated using MTS assay (CellTiter 96[®] AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) following the manufacturer's instructions. In brief, HDFs up to passage 10 or B16F10 melanoma cells were seeded at a density of 10,000 cells/cm² and allowed to attach to the well plate overnight. Particle suspensions in various known concentrations (0–500 µg ml⁻¹) were added to the cells and incubated for 24 hours. Cells exposed to media only served as controls. Similarly, the toxicity of free drugs (combination of curcumin and doxorubicin) and drugs-loaded MBCSP on B16F10 melanoma and 3T3 fibroblasts was evaluated by keeping drugs concentrations (0–50 µg ml⁻¹) the same in all the groups. The effect of temperature was also analyzed by incubating drugs-loaded MBCSP at 37°C and 41°C. MTS assays were carried out and cell survival in experimental groups was calculated relative to that of the control.

2.6. Cellular uptake of particles

In order to investigate the cellular uptake kinetics of particles, 3T3 fibroblasts and B16F10 melanoma cells were seeded in culture well plates and incubated for 24 hours. The particle suspensions of known concentrations (0–500 µg ml⁻¹) were added to the cells and incubated for 6 hours (the optimal time for particle uptake by melanoma cells determined in pilot studies). After incubation, the particle solution was removed and the cells were washed several times with PBS. Cells were then lysed with 1% Triton in PBS for one hour. The iron and DNA content in the cell lysates were analyzed using iron and Pico-Green DNA assays, respectively, as described elsewhere [24]. The amount of iron was then normalized to the DNA content for each cell lysate. Further, to investigate the uptake mechanisms of particles by B16F10 cells, various endocytic inhibitors were used to inhibit specific pathways of cellular uptake. B16F10 cells, seeded in tissue culture plates as described above, were incubated with chlorpromazine (10 µg ml⁻¹) to inhibit clathrin vesicles formation, filipin III (1 µg ml⁻¹) to hinder caveolae, and amiloride (11.5 µg ml⁻¹) to inhibit macropinocytosis as previously described [25]. After one hour incubation, the cells were treated with a suspension of MBCSP or GRGDS-MBCSP (300 µg ml⁻¹) in presence of respective inhibitors for an additional hour of incubation. The extent of particle uptake was then quantified as described above.

2.7. In vitro MRI

Imaging phantoms were prepared by dissolving agarose (0.5% w/v) in warm water and adding 0.3 or 0.6 mg ml⁻¹ MBCSP. The samples were immediately transferred to 4°C to allow gelation of the agarose. Further, B16F10 melanoma cells were cultured as described earlier, and MBCSP (0.6 mg ml⁻¹), MBCSP without magnetite (0.6 mg ml⁻¹), PLGA particles (0.3 mg ml⁻¹), or PNIPAAm-AAm-AH particles (0.3 mg ml⁻¹) were added to the cells and incubated for two hours to allow cellular uptake. The medium was then removed and the cells were gently rinsed twice with PBS. Cells were then trypsinized, centrifuged, and resuspended in agarose solution to form the phantoms. The imaging phantoms containing melanoma cells only or PLGA particles only were also prepared as controls. All the phantoms were imaged using a 4.7 T Varian small animal MRI scanner (Varian, Palo Alto, CA) as described elsewhere [16]. Multi-slice T2-weighted imaging was performed (TR=2s, TE=15ms, FOV=30mmx30mm, slice thickness=2mm) with spin echo pulse sequence. The MR images were then analyzed in Matlab (Mathworks Inc., Natick, MA) and percentage drops in the MR signal intensities of the T2-weighted images of samples, compared to that of control, were calculated.

2.8. In vivo tumor targeting and biodistribution

To assess magnetic targeting of the GRGDS-MBCSP to tumors, an orthotopic mouse model using B16F10 melanoma cell line was chosen. The animal model was carried out using C57BL/6 female mice (6–10 week old) from Jackson Laboratory (Bar Harbor, ME, USA). The animal experiments were approved by the University of Texas at Arlington's Animal Care and Use Committee. The particles were loaded with NIR-797 (Sigma-Aldrich, St. Louis, MO), a fluorescent dye, for *in vivo* imaging. Subcutaneous tumor implantation of B16F10 was induced in the left flank of mice by injection of $2 \times 10^6/0.1$ ml cells suspended in PBS. Tumor size was continuously monitored by caliper measurement weekly to determine the tumor development. Once the tumor reached approximately 300 mm^3 , the animals were induced with 1% isoflurane inhalation anesthesia and GRGDS-MBCSP suspension ($100 \mu\text{l}$ of 5 mg ml^{-1}) was injected intravenously as previously described [26]. A magnetic disk of 2451 Gauss strength was applied directly onto the tumor area for two hours following the particle administration. *In vivo* monitoring of animals and detection of the particles was performed at 0, 1, 4, and 24 hours after particle injection using an *in vivo* Kodak imaging system (Carestream Health Inc., Rochester, NY).

2.9. Statistical analysis

Results obtained were analyzed using one way ANOVA with $p < 0.05$ and Post-hoc comparisons (GraphPad Prism version 5.02 for Windows). All the experiments were repeated multiple (at least two) times with sample size of four. All the results were presented as mean $\pm$ standard deviation.

3. Results and discussion

3.1. Characterization of MBCSP

The amount of magnetite encapsulated into the MBCSP was estimated by iron assays, which was 3% (w/w) when bare magnetite was used and 16% (w/w) when VTMS-functionalized magnetite was used to make MBCSP. Results demonstrate that compared to bare magnetite, silane coupling of iron oxide nanoparticles allows more iron content (5-fold increase) to be included in MBCSP. It has been well illustrated that the magnetite without any coating have a very high tendency to aggregate [19]. Functionalization of magnetite with thiols, silica, oleic acid, and others has made them more dispersible and stable in aqueous solutions [19]. We speculate that the presence of thiol groups from silane coupling reagents improves the interaction of the magnetite and the polymer core during encapsulation as observed in previous studies [27]. Therefore, VTMS-functionalized magnetite was used for later studies.

As shown in a schematic representation of MBCSP (Fig. 1A), the PLGA-magnetite core surrounded with a thermo-responsive polymer shell formed a core-shell structure. TEM images of particles show that the MBCSP were 300–350 nm in size (Fig. 1B). The presence of the magnetite (black dots) throughout the PLGA core (solid white core) was evident. The presence of PNIPAAm-AAm-AH polymer shell surrounding the particles was also clearly observed as a grey surrounding. The hydrodynamic size, polydispersity index, and zeta potential of the particles were measured by DLS (Table 1). The results show that the mean hydrodynamic diameter of the MBCSP was approximately 296 nm with a polydispersity index of 0.28 and zeta potential of -12.07 mV .

The chemical composition of the particles was determined using FTIR spectroscopy (Fig. 1C). As shown in FTIR spectra of PLGA magnetite, the peak at 3496.2 cm^{-1} corresponds to OH stretching, due to the presence of the carboxyl functional group on PLGA-magnetite particles. The sharp and intense peak at 1750 cm^{-1} is a characteristic peak of the carbonyl group of ester. The peaks in the range of $700\text{--}800 \text{ cm}^{-1}$ correspond to the vinyl double bond

stretching due to the presence of VTMS-functionalized magnetite into the PLGA particles. Further, in FTIR spectra of AH-functionalized PLGA-magnetite particles, the peaks in the range of 1550-1650 cm^{-1} represent the coupling of the C=OH bonds present on the surface of PLGA with AH ($-\text{NH}_2$) to form an amide bond ($\text{C}=\text{ONH}_2$). Finally, the conjugation of PNIPAAm-AAm-AH to PLGA-magnetite core intensifies the OH stretching bond at 3500 cm^{-1} , due to polymer hydration in the FTIR spectra of MBCSP.

The magnetic properties of MBCSP were assessed using a SQUID magnetometer. Saturation magnetization of MBCSP was reduced in comparison to bare magnetite due to diamagnetic moment generated from the polymeric shell (Fig. 2). This observation is in agreement with previous reports where a polymeric barrier caused reduction in saturation magnetization of magnetic nanoparticles [20, 28]. Although the polymer coating reduced magnetic properties of MBCSP to 15.7 emu g^{-1} , it is still within the range for magnetic drug targeting applications [29, 30]. On the other hand, coercivity of the MBCSP was similar to that of bare magnetite, as it is negligible in the absence of an external magnetic field (Table 2). In addition, remanence was minimal after an applied low magnetic field, indicating the superparamagnetic behavior of MBCSP, which is similar to that of bare magnetite.

3.2. Drug loading and release from MBCSP

The loading efficiency of curcumin, in the core of MBCSP, was approximately 68%. There was negligible release of curcumin during the first 72 hours (Fig. 3A), followed by a burst release until day 12, and a sustained release until day 25. About 78% of the loaded drug was released over 25 days at 41°C. Moreover, there was no significant difference in the curcumin release at temperatures of 25°C and 41°C, indicating that the curcumin release was dependent on degradation of PLGA and not on the changes in temperatures. In addition to degradation, poor solubility of hydrophobic curcumin in PBS might have contributed to the prolonged release of curcumin. Previous studies also observed a sustained release of drugs including doxorubicin from PLGA nanoparticles over a period of 12 days [31]. The negligible release of curcumin during the first 72 hours might be due to the thermo-sensitive polymer shell that may have hindered the degradation of PLGA core, leading to a more sustained release of curcumin in comparison to that of PLGA particles alone.

The loading efficiency of doxorubicin, in the thermo-sensitive polymer shell of MBCSP, was approximately 60%. As shown in Fig. 3B, the doxorubicin release was significantly higher at 41°C (above LCST) than at 25°C (below LCST), establishing the temperature-sensitive behavior of the polymer shell. The releases at both temperatures exhibited a similar trend with the burst release occurring during the first 24 hours. The burst release of drugs at 25°C might be due to the presence of some doxorubicin on the surface of the particles. The total drug released over 25 days at 25°C and 41°C was about 58% and 80%, respectively. Thus, the temperature-dependent drug release characteristics suggest the potential use of MBCSP for controlled drug release in response to changes in temperature.

Although the detailed mechanism of drug release has yet to be determined, we believe that the PLGA degradation rates, the hydrophobicity of drugs and polymers, and the interactions of loaded drugs with polymers play an important role in determining the drug release rates in our system. At 25°C, the shell presents hydrophilic characteristics. The hydrophobic curcumin from the core can easily diffuse through the hydrophilic shell, while the release of hydrophilic doxorubicin slows down. On the other hand, at 41°C, the shell becomes hydrophobic. The release rate of curcumin slows down, and this might be due to curcumin's affinity to hydrophobic shell. Nevertheless, the results demonstrate that MBCSP consisting of a thermo-sensitive polymer shell on the PLGA-magnetite core could provide dual drug release mechanisms: controlled drug release in response to changes in temperature and sustained drug release via hydrolysis of biodegradable PLGA.

3.3. In vitro cytotoxicity and cellular uptake of particles

The cytocompatibility of MBCSP and GRGDS-MBCSP was assessed by the HDFs survival. HDFs exposed to particles with concentrations of 500 $\mu\text{g ml}^{-1}$ or below showed an 85–90% survival (Fig. 4A). These results suggest that MBCSP and GRGDS-MBCSP are relatively cytocompatible to fibroblasts. The polymer-coated magnetite particles thus overcome one of the main limitations of bare magnetite being toxic, especially at high concentrations [32]. Conjugation of GRGDS peptides to MBCSP improved the cytocompatibility of particles in healthy cells and enhanced the uptake of particles by melanoma cancer cells as shown in our studies. The cytocompatibility results are consistent with earlier findings on different magnetic-based thermo-sensitive particles from our group [16]. Further, free drugs and drugs-loaded MBCSP were exposed to B16F10 cells and 3T3 fibroblasts. As shown in Fig. 4B, both free drugs and drugs-loaded MBCSP were toxic to the melanoma cells, but not to the 3T3 fibroblasts, especially up to 25 $\mu\text{g ml}^{-1}$ (Fig. 4C). The high uptake of drug-loaded MBCSP by the B16F10 cells might account for the high toxicity to this cell type.

To assess the cancer targeting property, particles were incubated with off-target 3T3 fibroblasts and target B16F10 melanoma cancer cells. Indeed, the results show that there is a dose-dependent uptake of particles by the cells. The particle uptake by the 3T3 cells was relatively low compared to uptake by B16F10 cells (Fig. 5A). Moreover, there was no significant difference between MBCSP and GRGDS-MBCSP uptake by 3T3 fibroblasts. However, the uptake of GRGDS-MBCSP was significantly higher than that of MBCSP by B16F10 cells, due to further reinforcement in the cellular uptake by the GRGDS peptides (Fig. 5B). Similarly, Wang et al. [31] found that doxorubicin-loaded PLGA-PEG-cRGD nanoparticles were taken up by the $\alpha_5\beta_3$ integrin expressing cells in significantly higher amount than that of PLGA-PEG nanoparticles and doxorubicin alone. The mechanism by which MBCSP and GRGDS-MBCSP were internalized by melanoma cancer cells was also identified using the endocytotic inhibitors that blocked the activity of caveolae buds, clathrin coated pits, and macropinocytotic pathways. We find that the cellular uptake of MBCSP and GRGDS-MBCSP exhibited a 60% and 80% reduction, respectively, when the caveolae pathways were blocked (Fig. 5C). Although other inhibitors also blocked the cellular uptake of the particles, caveolae pathways play the most important role in the cellular uptake of GRGDS-MBCSP. In addition, the least inhibition, when all three inhibitors were used, may be attributed to either the un-synergistic effects of all three inhibitors or some different pathways might also be involved in the particle uptake.

3.4. In vitro MRI

In addition to controlled drug delivery, MBCSP can be used as contrast agents for MRI. The MRI on agarose phantoms containing PLGA particles display a bright MR signal (Fig. 6A) while MBCSP exhibit a darker MR signal (Fig. 6B–C). Furthermore, the negative contrast generated is greatest for the highest concentration of MBCSP (Fig. 6C). In order to test the usefulness of MBCSP as MRI contrast agents when internalized by cancer cells, the agarose phantoms containing MBCSP within melanoma cancer cells were also analyzed. Results show more intense negative contrast from the particles uptaken by the cells (Fig. 6H) compared to that of the cells only (Fig. 6D), and cells uptaken PLGA core (Fig. 6E), PNIPAAm-AAm-AH shell (Fig. 6F), or MBCSP without magnetite (Fig. 6G). The optimal concentration of MBCSP, showing effective uptake by the melanoma cells, also gave a more dispersed negative contrast signal compared to MBCSP alone. Moreover, percentage drops in the MR signal intensities of the samples was calculated (Fig. 6I), which demonstrated that the MBCSP uptaken by B16F10 cells generated darkest negative contrast and dropped MR signal intensity by 86.32% compared to 28.14% in case of MBCSP (0.6 mg ml^{-1}) alone. Interestingly, MBCSP uptaken by melanoma cells generated darker negative contrast than MBCSP at 0.6 mg ml^{-1} concentration, which might be due to a more dispersed signal in the

case of MBCSP uptaken by cells, creating the entire image in the gray shade. MRI results are in agreement with other published work [33] and our previous work [16], demonstrating the potential use of magnetic-based particles for non-invasive tracking of tumors.

3.5. In vivo tumor targeting

Preliminary *in vivo* studies were carried out using a melanoma orthotopic model in the thigh of mice. Optical images of control tumors (targeted particles without applying a magnetic field) (Fig. 7A) exhibited passive accumulation of GRGDS-MBCSP only for certain periods of time (1–4 hours). 24 hours post particle administration, the signal was not detectable (Fig. 7B). Whereas in the experimental tumors (targeted particles in the presence of a magnetic field), GRGDS-MBCSP were exclusively accumulated near the tumor where the magnet was applied at the tumor site for the initial two hours, even after 24 hours of particle administration. It is likely that the accumulation of particles can be further enhanced with prolonged or repeated applications of magnetic field at the tumor site. Wu et al. [34] observed that dextran-coated magnetite nanoparticles were accumulated at the right kidney, where the magnetic field was applied, in a significantly higher amount compared to other organ tissues. Moreover, Danhier et al. [5] observed that GRGDS-conjugated PLGA nanoparticles were more significantly targeted to tumors compared to non-conjugated nanoparticles. Our preliminary *in vivo* results demonstrate good targeting characteristics of MBCSP, making them a potential candidate for melanoma cancer therapy in the future.

In conclusion, we focused on the development of magnetic-based core-shell particles for providing controlled release of anti-cancer drugs via dual release mechanisms: temperature- and degradation-dependent. We successfully developed the particles and characterized their properties for use in targeted drug delivery and imaging applications for melanoma cancer therapy. The encapsulation of magnetite in the biodegradable polymeric core reduces toxicity and aggregation issues associated with bare magnetite, while preserving their magnetic properties. The presence of the biodegradable core also represents an efficient vehicle for sustained drug release applications for a long-term cancer treatment, while the thermo-responsive polymer shell provides rapid release of drugs in response to changes in temperature, which can be used along with hyperthermia treatment. The particles exhibited excellent cytocompatibility to healthy cells and efficient uptake by the targeted cancer cells. Moreover, the particles displayed a high potential as imaging probes for MRI and optical imaging modalities. Finally, the tumor-targeting capabilities of GRGDS-conjugated particles are promising, as they are effectively recruited by static magnets to the tumor site in melanoma mice models.

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Fig. 1. Characterization of MBCSP. A) Schematic of MBCSP. B) TEM image of MBCSP (Scale bar: 5000 nm); inset shows core-shell structure of MBCSP (Scale bar: 200 nm). C) FTIR spectrum of 1) PLGA-magnetite nanoparticles, 2) AH-functionalized PLGA-magnetite nanoparticles, and 3) MBCSP.

Fig. 2. Magnetic hysteresis loops of bare magnetite and MBCSP showing superparamagnetic behavior.

Fig. 3.

Drug release profiles of MBCSP. A) Curcumin release from core of MBCSP at 25°C and 41°C, showing sustained release profiles. B) Doxorubicin release from shell of MBCSP at 25°C and 41°C, showing temperature-dependent release profiles.

Fig. 4. Cytotoxicity of particles after 24 hours of exposure. A) Cytocompatible MBCSP and GRGDS-MBCSP with HDFs, showing no significant cell mortality up to 500 $\mu\text{g ml}^{-1}$ concentration. B) Toxicity of free drugs and drugs-loaded MBCSP on B16F10 cells and C) 3T3 fibroblasts at 37°C and 41°C, showing significant mortality in case of B16F10 cells. 3T3 fibroblasts survival was not reduced significantly up to 25 $\mu\text{g/ml}$ drug concentrations. * $p < 0.05$ as compared to controls.

Fig. 5.

Cellular uptake of MBCSP. A) Particle uptake by 3T3 fibroblasts showing no significant difference between uptake of MBCSP and GRGDS-MBCSP. B) Dose-dependent particle uptake by B16F10 cells showing higher uptake of GRGDS-MBCSP than MBCSP; * $p < 0.05$ as compared to MBCSP uptake. C) Cellular uptake of MBCSP and GRGDS-MBCSP by B16F10 cells using different endocytotic pathway inhibitors; * $p < 0.05$ as compared to untreated groups.

Fig. 6. MR images of agarose phantoms containing: A) PLGA nanoparticles (0.3 mg ml^{-1}), B) MBCSP (0.3 mg ml^{-1}), C) MBCSP (0.6 mg ml^{-1}), D) B16F10 cells only, and E) B16F10 cells exposed to PLGA core (0.3 mg ml^{-1}), F) PNIPAAm-AAm-AH shell (0.3 mg ml^{-1}), G) MBCSP without magnetite (0.6 mg ml^{-1}), H) MBCSP (0.6 mg ml^{-1}). I) MR signal intensity drop in showing highest drop in case of MBCSP (0.6 mg ml^{-1}) uptaken by B16F10 cells.

Fig. 7.

In vivo detection of GRGDS-conjugated MBCSP targeted to melanoma. A) In control animals without magnetic targeting, the signal of accumulated particles was intensely detected at early time points. However, the signal was rarely detected at 24 hours after particle administration. In treated animals, magnet was placed on top of tumor (red circle) of melanoma bearing animals. The signal of accumulated particles was detectable until 24 hours post injection. B) Quantification of fluorescence intensity from tumors showing higher accumulation of particles at tumor in presence of magnetic field.

Table 1

Hydrodynamic sizes, polydispersity indices, and surface charge of particles

Sample	Average Diameter (nm)	Polydispersity Index	Zeta Potential (mV)
PLGA-magnetite	233.9	0.11	−16.46
MBCSP	296.0	0.28	−12.07
GRGDS-MBCSP	398.7	0.34	−21.09

Table 2

Saturation magnetization, remanence, and coercivity of particles

Sample	Saturation Magnetization (M_s) emu/g	Saturation remanence (M_r)	Remanence (M_s/M_r)	Coercivity (H_C)
Bare magnetite	57.88	7.06	8.19	2
MBCSP	15.65	2.14	7.31	1