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Thermoresponsive core–shell magnetic nanoparticles for combined modalities of cancer therapy

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Abstract
Thermoresponsive polymer-coated magnetic nanoparticles loaded with anti-cancer drugs are of considerable interest for novel multi-modal cancer therapies. Such nanoparticles can be used for magnetic drug targeting followed by simultaneous hyperthermia and drug release. γ-Fe₂O₃ iron oxide magnetic nanoparticles (MNP) with average sizes of 14, 19 and 43 nm were synthesized by high temperature decomposition. Composite magnetic nanoparticles (CNP) of 43 nm MNP coated with the thermoresponsive polymer poly-n-isopropylacrylamide (PNIPAM) were prepared by dispersion polymerization of n-isopropylacrylamide monomer in the presence of the MNP. In vitro drug release of doxorubicin-(dox) loaded dehydrated CNP at temperatures below and above the lower critical solution temperature of PNIPAM (34 °C) revealed a weak dependence of drug release on swelling behavior. The particles displayed Fickian diffusion release kinetics; the maximum dox release at 42 °C after 101 h was 41%. In vitro simultaneous hyperthermia and drug release of therapeutically relevant quantities of dox was achieved, 14.7% of loaded dox was released in 47 min at hyperthermia temperatures. In vivo magnetic targeting of dox-loaded CNP to hepatocellular carcinoma (HCC) in a buffalo rat model was studied by magnetic resonance imaging (MRI) and histology. In summary, the good in vitro and in vivo performance of the doxorubicin-loaded thermoresponsive polymer-coated magnetic nanoparticles suggests considerable promise for applications in multi-modal treatment of cancer.

1. Introduction

Hepatocellular carcinoma (HCC), commonly known as liver cancer, is a deadly disease that has the sixth highest occurrence and the third lowest survival rate among commonly occurring cancers [1]. In 2002, the number of HCC cases reported worldwide was 626 000 with a survival rate of less than 5% [1]. A major reason for the low survival rate is that late detection of the disease [2] precludes surgical or thermoablative treatments that confer the best survival. Patients with advanced stages of HCC often also have cirrhosis and liver impairment, which limits the dosage in systemic chemotherapy treatments and leads to inadequate drug delivery at the tumor site. For this and other reasons, systemic chemotherapy in HCC has shown little or no survival benefits and it is also characterized by severe side effects and systemic toxicity [2]. However, if systemic toxicities with chemotherapy can be ameliorated, the probability for tumor regression and improved survival will
be greatly enhanced. The accurate and specific delivery of drugs directly to these tumors (drug targeting) to minimize side effects is thus highly desirable.

Magnetic drug targeting (MDT) is a novel solution for targeted delivery of drugs to tumors with minimal systemic toxicity [3–10]. In MDT, nanoparticles comprising of magnetic cores and polymeric shells loaded with drugs are injected into blood vessels near the tumor. An external magnetic field gradient applied at the tumor site localizes these core–shell particles at the tumor site enabling precise drug delivery; this procedure maximizes drug concentration at the tumor and minimizes systemic toxicity. A significant extension of MDT is the use of a polymeric component which responds to environmental stimuli such as temperature and/or pH. Such thermo-responsive polymers exhibit a lower critical solution temperature (LCST) above which they undergo reversible volume shrinkage due to expulsion of hydrophobically bound water from the polymer chains [11–14]. When cooled below the LCST, the polymer absorbs water and re-swells. Drug release can be controlled by manipulating the temperature of the polymer shell, resulting in higher release rates above the LCST and lower rates below the LCST [15–17].

In vivo studies [4–6] of MDT have shown positive results including complete remission of squamous cell carcinoma in rabbits treated with mitoxantrone-loaded magnetic nanoparticles [5]. A phase-I human clinical trial with 4′-epidoxorubicin indicated encouraging results on the physiological tolerance of MDT by patients [4].

Magnetic nanoparticles can also be used for hyperthermia treatment of cancer. Hyperthermia is the heating of cells in the range of 41–47°C. This heating causes preferential death of cancerous cells while sparing healthy cells [18–20]. When MNP are exposed to an alternating magnetic field (AMF) heat can be generated by Néel relaxation, Brownian relaxation and hysteresis losses [8, 21–23], hence MNP can be used as hyperthermia agents [24–34]. Conventional hyperthermia techniques such as radio-frequency, microwave, water bath and hepatic perfusion suffer from several serious drawbacks such as insufficient coverage, poor temperature control and distribution, risk of organ damage, etc [19, 32]. In vivo trials on animal models have highlighted the potential of MNPs for treatment of solid tumors by various magnetic hyperthermia techniques [30, 32–37]. Recent clinical trials on humans have also yielded promising results [27, 28, 38].

Randomized trials have also demonstrated the scope of hyperthermia to improve the outcome of radiotherapy and chemotherapy [18, 19]. Hyperthermia enhances the efficacy of certain anti-neoplastic drugs. This synergistic effect depends on the temperature and drug as well as other factors such as pharmacodynamic and pharmacokinetic interactions [18, 39–41]. Some anti-neoplastic drugs, e.g. doxorubicin (dox), show threshold behavior, their cytotoxicity substantially increasing above a specific temperature. Magnetically mediated hyperthermia retains the advantages of conventional hyperthermia while significantly reducing or eliminating its limitations [32]. Magnetic nanoparticle-based carriers can overcome the drawbacks of conventional drug delivery and hyperthermia modalities through drug targeting; targeting can be followed by concurrent hyperthermia and drug release to leverage synergistic effects. Controlled in vitro drug release from thermo-responsive magnetoliposomes and thermo-responsive polymer-coated MNP with and without an AMF have been reported [17, 42, 43]. In vivo trials on rats implanted with AH60C tumors showed complete tumor remission by repeated hyperthermia treatments with thermosensitive magnetoliposomes, without the use of any drugs [37].

In our work, the novel approach of using multi-functional magnetic nanoparticles as agents for combined drug targeting, drug delivery and hyperthermia that can potentially improve multi-modal cancer therapy is examined. Heat generated in the magnetic core upon AMF application (magnetic hyperthermia) is conducted from the core to the surrounding polymer and raises the temperature of the drug-loaded polymer shell above the LCST, triggering drug release.

The composite nanoparticles (CNP) used in this work consisted of iron oxide as the magnetic core and the thermo-responsive polymer poly-n-isopropylacrylamide (PNIPAM) [11–15, 44, 45] as the shell component. Due to the biocompatibility and attractive magnetic properties of iron oxide, both iron oxide and PNIPAM have been previously studied for biomedical applications [17, 46–49]. The ability to tune the LCST of PNIPAM using the appropriate copolymer is an additional advantage.

The targeting, drug release and hyperthermia behavior of magnetic core/polymer shell nanoparticles was studied. The in vitro hyperthermia and drug release behavior as well as in vivo magnetic targeting and magnetic resonance (MRI) visualization in an HCC animal model was examined. MNP of average sizes 14, 19 and 43 nm were synthesized, characterized and their in vitro hyperthermia performance evaluated. Particles with optimum magnetic properties and heating behavior were coated with PNIPAM to produce composite nanoparticles, which were subsequently loaded with the anti-neoplastic drug doxorubicin. The in vitro drug release of the dox–CNP particles with and without an AMF was investigated and an in vivo MDT trial was carried out. Our studies showed that in vitro CNP released drugs under hyperthermia conditions and could be targeted in vivo to HCC in a rat model.

2. Materials and methods

2.1. Synthesis of iron oxide magnetic nanoparticles

Iron oxide MNP were synthesized by a modified high temperature thermal decomposition method described in detail elsewhere [50]. In a typical experiment to synthesize 14 nm MNP, 10.8 g of iron chloride (FeCl3·6H2O, 120 mmol, Aldrich, 98%) and 36.5 g of sodium oleate (120 mmol, TCI, 95%) were dissolved in a solvent mixture of 80 ml ethanol, 60 ml distilled water and 140 ml hexane in a round-bottomed flask. The solution was heated to 70°C under reflux conditions and held for 4 h. At the end of the reaction, the dark red upper organic layer containing the iron oleate complex was washed several times with 30 ml of distilled water in a separatory funnel and dried under vacuum for 72 h to yield
a dark red, waxy solid. In a round-bottomed flask with a reflux condensation set-up, 36 g (40 mmol) of the iron olate complex from the above procedure and 5.7 g of oleic acid (20 mmol, Aldrich, 90%) were dissolved in 200 g of the organic solvent 1-octadecene (Aldrich, 90%). The mixture was heated to 320 °C at approximately 3.5 °C min⁻¹ in argon. Upon reaching the reaction temperature, slightly above the boiling point of 1-octadecene (b.p. 317 °C), a vigorous reaction occurred and the initial transparent red solution began to turn brownish-black. The mixture containing the MNP was aged at 320 °C for 2 h and cooled to room temperature. The nanoparticles were precipitated by adding 500 ml of ethanol and extracted from the liquid with the help of a permanent magnet. However, the synthesized particles were coated with oleic acid and residual organic solvent, both of which are not biocompatible. To evaporate these organic residues from the surfaces of the particles, in a departure from the procedure in [50], the particles were heated to 400 °C and the temperature maintained for 1 h. The MNP were washed several times with ethanol, acetone and distilled water, dried and stored under vacuum. 

2.3. Characterization

Drops of dilute suspensions of the uncoated MNP were placed on carbon-coated copper grids and dried under vacuum for transmission electron microscopy. Imaging was conducted using a JEOL 2010F TEM operating at 200 kV. ImageJ image processing software was used to measure particle size from the micrographs. X-ray diffraction patterns of vacuum-dried bare MNP powders were obtained with a Shimadzu 6000 x-ray diffractometer using Cu Kα radiation (λ = 0.154 nm).

The composition was identified by matching the powder diffraction patterns to reference files using the Shimadzu phase identification software. A LakeShore 7404 vibrating sample magnetometer was used to measure the magnetic properties at room temperature of the MNP and CNP; the applied field was in the range of 0-1 T (10 kG). The polymer content of the composite nanoparticles was determined via thermogravimetric analysis using a TA instruments Q500 TGA unit. The weight loss up to 600 °C at a heating rate of 20 °C s⁻¹ was used to determine the PNIPAM content.

2.4. In vitro hyperthermia

Bare MNP were dispersed in MilliQ water under ultrasonication to create ferrofluids with a concentration of 10 mg ml⁻¹. Glass bottles containing 5 ml of ferrofluid and thermally insulated with ceramic wool were placed within a six-loop water-cooled copper coil driven by an Inductelec A.C. generator (Sheffield, UK). The applied frequency was 375 kHz and the heating behavior of the ferrofluids was studied at a field strength (H₀) of 1.7 kA m⁻¹. The ambient temperature was 30 °C. A Luxtron MD600 fiber optic thermometry unit connected to a computer was used to measure the ferrofluid temperature. The specific absorption rate (SAR) of iron oxide particles was calculated from the following equation [22]:

\[
SAR = C = \left( \frac{\Delta T}{\Delta t} \right) \frac{\text{mass ferrofluid}}{\text{mass nanoparticle}}
\]

where C is the mass weighted heat capacity of the ferrofluid and (ΔT/Δt) the slope of the initial section of the temperature versus time curve.

2.5. Drug loading with doxorubicin

Typically, dox was loaded onto the CNP by incubating 315 mg of the dehydrated particles in 200 ml of dox-MilliQ water solution (conc. 0.18 mg ml⁻¹) for 16 h at 22 °C. After loading, the particles were separated by centrifugation for 10 min at 8000 rpm, dried and stored under vacuum. The post-loading dox concentration of the supernatant was determined by measuring the absorbance at 482 nm in a UV–visible spectrophotometer and comparing it to previously prepared reference plots of dox solutions. The drug loading of the dox-loaded CNP (dox-CNP) was measured by the reduction in the amount of dox in solution during the incubation process.

2.6. In vitro drug release without AMF

Drug release kinetics without an AMF was studied at temperatures of 24 °C (below the LCST of pure PNIPAM), 37 °C (body temperature, just above the LCST) and 42 °C (above the LCST). 5 ml of ferrofluids with a concentration of 2 mg ml⁻¹ were prepared in glass test tubes by ultrasonically dispersing dox-CNP powders in PBS (pH 7.4). The sealed test tubes were placed in preheated water baths and the temperature stabilized (±1 °C) at 24, 37 and 42 °C for 101 h. 1 ml of the release medium (free of nanoparticles) was extracted and replaced with fresh PBS at 0.75, 1.75, 3.75, 10.75, 26, 32.5,
50, 77 and 101 h. Dox content of the extracted solution was measured by UV–visible spectrophotometry and cumulative dox release calculated.

2.7. In vitro simultaneous drug release and hyperthermia

Ferrofluids of volume 5 ml and concentration 2.5 mg ml$^{-1}$ were prepared by ultrasonically dispersing dox-CNP in PBS (pH 7.4) at 37°C and subsequently tested for simultaneous drug release and hyperthermia in a typical hyperthermia cycle using the experimental set-up described in section 2.3. Magnetic field strength was controlled from 0.1 to 4 kA m$^{-1}$ to increase the temperature and then maintain it in the hyperthermia temperature range. After 30 min of hyperthermia exposure, the ferrofluid was allowed to cool down to 37°C. The particles were immediately separated by centrifugation and dox release was measured via UV–visible spectrophotometry by analyzing the supernatant.

2.8. In vivo magnetic drug targeting and MRI visualization

In vivo MDT and MRI was carried out at the Department of Experimental Surgery and the Radiology Department of Singapore General Hospital. Male buffalo rats (Charles River, USA) were laparatomized and Morris Hepatoma 7777 cells (ATCC, USA) were implanted into the liver to induce development of HCC. Two weeks after implantation, MRI of the abdomen of the rat was performed using a custom-built MRI coil in a Siemens MRI scanner (Magnetom Allegra) to confirm that the HCC had developed in the liver; this scan also served as the baseline pre-treatment scan.

3. Results

3.1. Physical properties

Particles of average size 14 nm (SD 3.5), 19 nm (SD 4.9) and 43 nm (SD 5) were synthesized by the modified high temperature decomposition method. The shape of the particles is irregular, as seen from the transmission electron micrographs in figure 1. The phase of the powders was determined to be $\gamma$-Fe$_2$O$_3$ (maghemite) from the x-ray diffraction patterns (figure 2). Peak breadth decreases with increasing particle size, while sharp and clearly defined peaks indicate the high degree of crystallinity of the particles. The nanoparticles were dark brown, consistent with the interpretation of the x-ray data that these nanoparticles are primarily $\gamma$-Fe$_2$O$_3$ and not Fe$_3$O$_4$, which is black. The nanoparticles also contained minor quantities of the $\alpha$-Fe$_2$O$_3$ phase. Analysis of the TGA data (figure 3) showed that the weight loss between 200 and 440°C corresponding to the PNIPAM content [52] of the composite nanoparticles is $\sim$6 wt%.

3.2. Magnetic properties

Room temperature magnetization curves of the particles are shown in figure 4. The saturation magnetization ($M_s$)
Figure 3. Weight loss versus temperature curve for the PNIPAM-coated composite nanoparticles, the polymer content is 6 wt% of the CNP.

Figure 4. Room temperature magnetization curves of 14, 19 and 43 nm uncoated iron oxide nanoparticles as well as PNIPAM-encapsulated 43 nm composite nanoparticles (CNP). $M_S$ increases with increasing size for the bare particles.

Figure 5. Heating characteristics of iron oxide nanoparticles dispersed in water and subjected to a 375 kHz, 1.7 kA m$^{-1}$ alternating magnetic field. Ambient temperature = 30°C. Ferrofluid concentration = 10 mg ml$^{-1}$, volume = 5 ml. Dashed horizontal lines indicate the hyperthermia range of 41–48°C.

3.3. In vitro hyperthermia

The in vitro heating behavior of the bare MNP ferrofluids of concentration 10 mg ml$^{-1}$ subjected to a 375 kHz, 1.7 kA m$^{-1}$ AMF is shown in figure 5. The 43 nm MNP exhibited the fastest heating, reaching 41°C in ~2 min. This temperature was reached by the 19 nm and 14 nm particles in 3 min and 3.5 min, respectively. The specific absorption ratio was calculated to be 42.8 W g$^{-1}$ for the 43 nm, 30.1 W g$^{-1}$ for the 19 nm and 22.4 W g$^{-1}$ for the 14 nm MNP.

3.4. Doxorubicin loading and release

A schematic overview of the dox–CNP synthesis process for the 43 nm MNP is shown in figure 6. First, the CNP are synthesized by polymerizing NIPAM monomers in the presence of 43 nm particles (figure 6(a)). For storage, the synthesized CNP are then dehydrated in vacuum below the LCST. To synthesize dox–CNP the dehydrated CNP are swollen below the LCST in an aqueous solution containing doxorubicin (figure 6(b)). These drug-loaded particles are then again dehydrated for storage and further experiments.

From the UV–visible spectrophotometry data at 482 nm (not shown) of the post-loading dox solution it was concluded that the dox content of the dox–CNP particles was 2.5 wt%.

The dox release profiles at 24, 37 and 42°C for dox–CNP particles in PBS are shown in figure 7. The inset is an expanded view of the release for the first 12 h. Cumulative dox release for 101 h at 24°C was 28.8% of loaded dox, increasing to 36.3% at 37°C and 41% at 42°C. Drug release occurs relatively rapidly until 10.75 h, with 24.7% release at 24°C, 27% at 37°C and 28.1% at 42°C, after which the release slows down. This effect is most prominent at 24°C.

3.5. In vitro simultaneous hyperthermia and drug release

The temperature versus time plot for a hyperthermia cycle of (a) heating, (b) temperature stabilization and (c) cooling in the absence of a magnetic field is shown in figure 8. With the field strength in the range of 0.1–4 kA m$^{-1}$ and a starting temperature of 37°C, the ferrofluid heated up rapidly, reaching
Figure 6. Schematic overview of (a) the composite magnetic nanoparticle preparation, (b) drug loading and (c) drug release processes.

Figure 7. In vitro drug release profiles of dox-loaded composite magnetic nanoparticles in phosphate buffer (pH 7.4) at 24 °C (T < LCST), 37 °C (T > LCST) and 42 °C (T ≫ LCST) plotted against time t. External magnetic field absent. Particle conc. = 2 mg ml⁻¹, vol. = 5 ml. Inset is an expanded view of release for the first 12 h.

Figure 8. Temperature plot from the in vitro simultaneous drug release and hyperthermia test of dox-loaded composite magnetic nanoparticles in a 375 kHz, variable strength alternating magnetic field. Field was switched off after 30 min of hyperthermia exposure. Ferrofluid conc. = 2.5 mg ml⁻¹, vol. = 5 ml. Dox released = 14.7% (48 μg) of loaded dox.

41 °C in just 50 s, yielding an SAR of 137 W g⁻¹. Temperature stabilization within the hyperthermia range was successfully attained by variation of the field strength. Switching off the AMF resulted in the ferrofluid cooling down to 37 °C in 16 min. Due to the higher field strengths, the heating rate was rapid even with a lower concentration of the ferrofluid (2.5 mg ml⁻¹ compared to 5 mg ml⁻¹ for the hyperthermia experiment performed at a field strength of 0.1–4 kA m⁻¹) (figure 4). 14.7% (48.6 μg) of the loaded dox was released after a total of 47 min, i.e. 30 min of exposure to hyperthermia temperatures (41–48 °C) and 17 min above body temperature (37 °C) during the heating and cooling stage. The results are in close agreement with the observed dox release at 37 °C (14.6%) and 42 °C (14.9%) for a release time of 45 min.

3.6. In vivo magnetic drug targeting and MRI

The dox-loaded composite nanoparticles were successfully targeted to the HCC in a buffalo rat model. Figure 9(a) is
the pre-injection MRI baseline scan of the rat, where the HCC is uniformly gray. After ferrofluid injection and guidance by an external magnet, the dox-loaded MNP were immobilized in the HCC as seen by the dark regions (figure 9(b)) and confirmed by histology (figure 9(c)). Staining of HCC tissue clearly revealed iron oxide deposits. Histology of tissues from surrounding organs such as the kidney and spleen did not show iron oxide deposits, suggesting that the dox-loaded particles were accurately targeted to the HCC and not systemically distributed.

### 4. Discussion

#### 4.1. Effect of particle size

The optimum particle size for *in vivo* applications depends on a number of factors, especially for multi-modal applications such as combined magnetic drug targeting and hyperthermia. For *in vivo* applications, smaller particle size helps in evasion [7, 53] of the reticulo-endothelial system (RES), the body’s immune system responsible for detection and clearance of foreign particles from circulation. If the particles are prematurely cleared from circulation due to coating with plasma proteins (opsonization), targeting at the tumor will not be achieved. RES evasion and circulation times can be enhanced by appropriate surface functionalization of the particles [7, 10, 53]. Particles that are smaller than 10 nm are well suited for tumor penetration, but are rapidly cleared by renal filtration and extravasation. Particles in the size range of 50–150 nm are typically cleared by the Kupffer cells of the liver and those that are larger than 200 nm are likely to be filtered by the venous sinuses of the spleen [7, 10].

For magnetic nanoparticles, smaller size usually results in lower saturation magnetization [54, 55] resulting in a decreased response to externally applied magnetic fields. This increases the difficulty of targeting tumors deep inside the body. Generation of large static or alternating magnetic fields with sufficient penetration depth is technically challenging, hence it is important to ensure that the MNP have the highest $M_S$ values possible. The power output from MNP in magnetic hyperthermia is also strongly dependent on $M_S$ [22]: higher $M_S$ values result in higher heating power. For magnetic hyperthermia, the theoretical ideal magnetic particle core size for heat generation is in the 10–20 nm range, which is lower than the ideal size for MDT, but larger than the ideal size for tumor penetration (approx. <10 nm). Hence, particles in the 10–50 nm size range, with appropriate coatings, provide a good balance of circulation times, ability to be manipulated by an external magnetic field and heat generation for hyperthermia.

Iron oxide nanoparticles of three average sizes—14, 19 and 43 nm—were synthesized by the modified high temperature thermal decomposition method (figure 1). To obtain particles free from organic residue an additional high temperature solvent evaporation stage for removal of organic components was incorporated in the synthesis. The particles showed polydispersity (standard deviation of 3.5–5) and irregular shape. The size distribution is partially due to the continuously decreasing volume of the residual organic solvent, resulting in a dynamically changing ratio of reactants and solvent, hence there is non-uniform particle growth. Aggregation of the particles is due to the absence of solvent and surfactants which reduce interparticle attraction (figure 1). In the dry state nanoparticles have a tendency to aggregate to reduce their surface area and surface charge, regardless of

**Figure 9.** MRI scan of the buffalo Rat implanted with HCC: (a) baseline scan before injection of dox-CNP and (b) 30 min after injection. Particles are seen as new dark regions in the HCC. (c) Histology slide of the HCC showing particles as dark deposits. (This figure is in colour only in the electronic version)
magnetic properties. When the particles are coated with a polymer and dispersed in a carrier fluid, the aggregation is reduced.

The high temperature synthesis conditions yielded highly crystalline particles especially for the 19 and 43 nm MNP, as evidenced by the sharp peaks of the XRD patterns (figure 2). Prior to the evaporation stage, the particles are expected to be Fe3O4 (magnetite) but due to exposure to air during solvent evaporation and further processing, they are converted to γ-Fe2O3 (maghemite). Nanoparticles have a very large surface area to volume ratio, promoting oxidation to more stable states, especially at higher temperatures. In the nanosize range, γ-Fe2O3 is more stable than Fe3O4 [56] and without a protective surface coating retention of magnetite nanoparticles in their native form is difficult. Both Fe3O4 and γ-Fe2O3 are suitable for in vivo biomedical applications [4, 29, 30, 32–35].

4.2. Magnetic properties of the composite nanoparticles

The magnetic properties of the MNP were strongly size-dependent (figure 4) with the 43 nm average-sized MNP exhibiting the highest $M_s$ of 267 kA m$^{-1}$ followed by the 19 nm MNP at 207 kA m$^{-1}$ and the 14 nm MNP at 115 kA m$^{-1}$. As expected these values are lower than the bulk $M_s$ of maghemite of ~414 kA m$^{-1}$ [55]. One reason could be the spin disorder in the surface layers of the MNP [54], their high surface area to volume ratio magnifying this effect as size decreases. $H_C$ also increases with particle size, with the lowest value of 1.1 mT exhibited by the 14 nm MNP and $H_C$ increasing to 7 mT for the 43 nm particles. Below a particle size of ~166 nm, γ-Fe2O3 particles are single domain; $H_C$ decreases with particle size until the superparamagnetic limit when it becomes zero. It is likely that superparamagnetic particles are present within the size distribution for the 14 and 19 nm average size MNP. However, due to the presence of larger particles, measured coercivity values are higher. The 43 nm MNP are not superparamagnetic at room temperature, yet well below the single-domain limit. Upon conversion of the 43 nm MNP to PNIPAM–iron oxide composite nanoparticles, magnetic properties are largely preserved, with a 32 kA m$^{-1}$ drop in $M_s$. Thus the particles are suitable for in vivo applications.

4.3. Hyperthermia

In vitro hyperthermia tests (figure 5) of the bare MNP indicate good heating properties, especially for the 43 and 19 nm particles, with the highest SAR value of 42.8 W g$^{-1}$ exhibited by 43 nm MNP for an applied field of 1.7 kA m$^{-1}$. The ferrofluid temperature rose quickly in the case of 43 nm MNP, crossing 41 °C in slightly over 2 min despite the low field strength and moderate frequency. This in vitro test is a useful guide to the performance of the particles although in vivo heating will be altered due to factors such as tissue thermal conductivity, cooling of the tumor by perfusion and heat loss from surrounding tissues. It is important to maximize the power output of the particles to achieve therapeutic temperatures in the tumor with the minimum quantity of particles. In vivo conditions favor hyperthermia for larger HCC tumors; although particle deposition in ‘large’ tumors was lower than in ‘small’ tumors, heating rates in larger tumors were higher due to poorer cooling effects from low blood perfusion and better heat conduction from tumor vasculature structure [33].

The dominant mechanisms for heat generation from the 14 nm MNP are Néel and Brownian relaxation processes [22], while hysteresis losses are more important for the 19 nm and 43 nm MNP particles which are not superparamagnetic [21, 23]. It has been suggested that single-domain particles in the transitional size range between single domain and superparamagnetic, e.g. 30–40 nm bacterial magnetosomes [21], may produce higher power output than purely superparamagnetic particles, but this is also dependent upon specific field parameters and environmental conditions. Heating rates and power output for the 14 nm MNP can be further increased by increasing the applied field strength. For superparamagnetic particles the power output is proportional to the square of the field [21–23].

In the case of single-domain particles such as the 43 nm MNP, minor saturation loops are produced with a 1.7 kA m$^{-1}$ AMF; greater heating can be achieved at higher field strengths by fully saturated hysteresis loops. The ferrofluid concentrations (10 mg ml$^{-1}$) used for our in vitro tests are conservative compared to reported literature values; for example, more than 100 mg ml$^{-1}$ used in recent clinical trials [27] and 20–50 mg ml$^{-1}$ in animal hyperthermia and MDT trials [5, 33, 35].

4.4. Drug loading

From the TGA curve (figure 3), the PNIPAM content of the CNP was estimated to be 6 wt%. Different synthesis techniques have been reported [17, 48, 49, 51] for the preparation of thermoresponsive magnetic particles. The dispersion polymerization method was chosen in this study because it is a relatively benign procedure which does not require the use of surfactants which can alter the LCST [11]. In dispersion polymerization, both heterogeneous and homogeneous nucleation can occur with nucleation and growth of the polymeric particles occurring around iron oxide nanoparticle ‘seeds’ [51]. Coordination between iron oxide ions and surface hydroxy groups to the free ion pair of amic acid nitrogen in NIPAM aids adsorption of the monomer to the nanoparticle surface, producing clusters of iron oxide MNP with a coating of PNIPAM (figure 6(a)).

Dehydration of the CNP in vacuum at temperatures less than the LCST of PNIPAM in water (~32–34 °C) should cause shrinkage of the polymer layer. When these dehydrated particles are swollen below the LCST in an aqueous solution of a water-soluble drug there is uptake of the drug molecules into the polymer matrix along with the solvent (figure 6(b)). Drug loading can be achieved in two ways: (a) the drug is loaded onto the particles in a separate step post-synthesis by incubating the composite particles in a drug solution below the polymer LCST or (b) the drug is added to the reaction mixture at the synthesis step before polymerization to entrap the drug molecules within the polymer matrix [57]. The latter method
may cause undesirable changes in the properties of the drug due to the presence of other reactants, hence method (a) was chosen in this work.

The dox loading achieved was 2.5% of the total weight of the dox-loaded CNP. This is sufficient to achieve dox release during in vitro simultaneous hyperthermia and drug release in the range of μg with conservative concentrations of particles. If necessary, the loading can be increased by increasing the concentration of the dox solution or the incubation time.

4.5. Drug release

For drug release from dox-loaded CNP in PBS at 24°C, 37°C and 42°C in the absence of an external magnetic field, there is an initial rapid release phase for times of up to ~10.75 h by which time the particles release 24.7%, 27% and 28.1% of the loaded dox at 24°C, 37°C and 42°C, respectively (figure 7). This is followed by slow release behavior up to 101 h, with cumulative dox release of 28.8% (24°C), 36.3% (37°C) and 41% (42°C).

Drug release from dehydrated hydrogels is Fickian at temperatures above the LCST [11], i.e. 37°C and 42°C, as schematically depicted in figure 6(c)-i. Below the LCST (<34°C) the PNIPAM matrix is expected to slowly swell and, due to changes in the pore size, drug release should decrease (figure 6(c)-ii). This explains the similar initial release behavior observed for all three temperatures, with a deviation expected after the equilibrium swelling state of PNIPAM is reached at 24°C. However, even after more than 10 h, similar release profiles are obtained for dox release in the temperature range of 24–42°C. The PNIPAM matrix is unable to actively regulate the transport of doxorubicin molecules due to a large difference in the matrix pore size and the size of the drug molecule. Triggered drug release utilizing the thermoresponsive behavior is not feasible from particles in the dehydrated state. Vacuum dehydration appears to result in structural changes to the PNIPAM, depressing its re-swelling behavior. On the other hand, recent results on swollen (hydrated) drug-loaded composite particles of ~13 nm average size Fe3O4 MNP coated with PNIPAM (to be reported elsewhere) confirm pronounced thermoresponsive behavior. The swollen particles dispersed in PBS and tested at 24, 37 and 43°C exhibited clear thermoresponsive behavior with higher release rates above the LCST (78.1% at 43°C versus 42.6% at 24°C after 29 h). Significantly, we were able to modulate drug release by cycling the temperature below and above the LCST, thus using the LCST as an on/off switch. Drug release dropped to nearly zero below the LCST and resumed above the LCST.

A clear difference in release rates of 5-fluorouracil from dextran-magnetite-incorporated thermosensitive liposomes at temperatures in the range of 37°C to 42°C with and without an applied AMF was reported earlier with a different experimental protocol by Viroonchatapan et al [42]. Recently, Zhang et al [17] have shown controlled release of doxorubicin from core–shell nanoparticle carriers comprising of an iron oxide core and a thermoresponsive polymer shell in the temperature range of 20–40°C, without an applied magnetic field. The dox was chemically bonded to functionalized iron oxide nanoparticle cores and encapsulated with the polymer; in contrast, our approach involves loading the polymer shell with the drug. Our in vitro experiments demonstrate that drug release of therapeutic amounts of dox can be achieved by AMF-induced heating of the dox–CNP ferrofluid. The synergistic effect of heat enhancing the cytotoxicity of the drugs [18, 40, 41] may be realized following magnetic drug targeting to the tumor.

4.6. In vivo experiments

In in vivo MDT experiments, reported in detail elsewhere [58], dox–CNP deposition in the HCC was confirmed by MRI (figure 9(b)) as well as by histology of the HCC sections (figure 9(c)). The ferrofluid volume (0.5 ml) and concentration (0.36 mg ml−1) injected were optimized for MRI visualization in order to track the particles in vivo. For hyperthermia applications, the particle concentration should be increased significantly to produce therapeutic temperatures. While normal liver tissues obtain about 70% of their blood supply from the portal venous system, the hepatic arterial system is the main source of blood supply for liver tumors. This makes preferential targeting of liver tumors via the hepatic artery much easier [33]. The absence of particle deposits in healthy liver tissue or other organs of the buffalo rat supports this assertion.

In summary, composite nanoparticles consisting of drug-loaded thermoresponsive polymer-coated magnetic nanoparticles were synthesized and studied for hyperthermia, drug release and drug targeting applications in cancer therapy. These studies showed significant in vitro drug release under hyperthermia conditions, in vivo magnetic drug targeting was also demonstrated.

5. Conclusions

- In vitro hyperthermia and drug release, as well as in vivo magnetic targeting and imaging studies on thermoresponsive magnetic nanoparticles for combined drug targeting, delivery and hyperthermia applications were carried out. Magnetic nanoparticles of γ-Fe2O3 (average size 14, 19 and 43 nm) were synthesized, characterized and their heating behavior in an alternating magnetic field was studied. The 43 nm particles had the best magnetic properties and heating power output.
- Composite nanoparticles of 43 nm γ-Fe2O3 particles coated with the thermoresponsive polymer poly-n-isopropylacrylamide (PNIPAM) were prepared and loaded with the anti-cancer drug doxorubicin.
- Simultaneous hyperthermia and drug release in vitro was exhibited by these dehydrated particles dispersed in phosphate buffer solution (PBS) when exposed to an alternating magnetic field. In vitro drug release tests from dehydrated particles in PBS, below and above the LCST of PNIPAM in water, showed little differences in drug release rates. However, recent results (to be published elsewhere) confirm that swollen (hydrated) particles tested under similar conditions clearly exhibit thermoresponsive behavior.
• In vivo magnetic targeting to the tumor with dox-loaded PNIPAM–iron oxide nanoparticles was studied in a buffalo rat model implanted with hepatocellular carcinoma (HCC). Magnetic resonance imaging (MRI) and histology showed that the injected particles were localized in the HCC.

• The in vitro dox release under magnetic hyperthermia conditions and in vivo magnetic drug targeting with these novel multi-functional particles suggests considerable promise for applications in the multi-modal treatment of cancer.

References

[16] Liu S Q, Tong Y W and Yang Y T 2005 Incorporation and in vitro release of doxorubicin in thermally sensitive micelles made from poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide)-b-poly(d,l-lactide-co-glycolide) with varying compositions Biomaterials 26 5064–74
[34] Moroz P, Jones S K and Gray B N 2002 Tumor response to ferromagnetic embolization hyperthermia in a rabbit liver tumour model Int. J. Hyperth. 18 129–40
[40] Takemoto M et al 2003 The effect of various chemotherapeutic agents given with mild hyperthermia on different types of tumours Int. J. Hypertherm. 19 193–203
[41] Urano M, Kuroda M and Nishimura Y 1999 For the clinical application of thermochemistry given at mild temperatures Int. J. Hypertherm. 15 79–107