International Journal of Pharmaceutics 424 (2012) 107–114

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

Pluronic@Fe_3O_4 nanoparticles with robust incorporation of doxorubicin by thermo-responsiveness

Shinyoung Park[a,1], Hye Sung Kim[a,1], Woo Jin Kim[b], Hyuk Sang Yoo[a,c,*]

[a] Department of Biomaterials Engineering, Kangwon National University, Chuncheon 200-701, Republic of Korea
[b] Department of Internal Medicine, School of Medicine, Kangwon National University, Republic of Korea
[c] Institute of Bioscience and Bioengineering, Kangwon National University, Republic of Korea

ARTICLE INFO

Article history:
Received 7 July 2011
Received in revised form 5 December 2011
Accepted 25 December 2011
Available online 31 December 2011

Keywords:
Magnetic nanoparticle
Doxorubicin
Temperature-sensitive
Pluronic
Imaging

ABSTRACT

Doxorubicin was physically incorporated in magnetic nanoparticles by thermo-responsive manners. Magnetic nanoparticles were prepared by oxidizing ferric ions in ammonium solution. Thiolated Pluronic was synthesized by sequential modification of terminal hydroxyl groups of Pluronic to amine groups and thiol groups. Magnetic nanoparticles composed of iron oxide were surface-modified with thiolated Pluronic at different molar ratios of iron to thiol groups. Pluronic decoration on the magnetic nanoparticles was characterized by elemental analysis and transmission electron microscopy. Elemental analysis results on carbon atoms in the magnetic nanoparticles showed that the degree of Pluronic decoration was proportional to the feed ratio of thiolated Pluronic to iron oxide. Doxorubicin was incorporated to the magnetic nanoparticles thermo-responsive manners; a mixture of hydrophobized doxorubicin and the magnetic nanoparticles was incubated at 4 °C and the temperature was subsequently increased to 37 °C for thermally induced structural changes of the decorated Pluronic moieties. Doxorubicin-incorporated magnetic nanoparticles showed dramatic modulations of size distributions according to temperature changes, which was dependent on the degree of Pluronic decoration. Loading efficiency of doxorubicin was significantly affected by the number of decorated Pluronic on the magnetic nanoparticles; the higher Pluronic moieties the nanoparticles had, the higher loading efficiency they showed. Release profiles of doxorubicin from the nanoparticles showed that doxorubicin was liberated from the nanoparticles in response to reducing conditions of the release medium. Anti-cancer activities of the doxorubicin-incorporated nanoparticles were determined by a MTT-based cytotoxicity assay against A549 cell lines. Compared to native doxorubicin, the doxorubicin incorporated magnetites showed attenuated cytotoxicities due to slow release of doxorubicin from the carriers. Thus, thermally induced incorporation of anti-cancer drugs can be a novel method for multifunctional magnetic nanoparticles with imaging and anti-cancer treatments.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Magnetic nanoparticles have been extensively prepared for the potential use of a burgeoning field of nanomedicine such as imaging and drug delivery (Brzozowska and Krysinski, 2009; Yallapu et al., 2011; Mejías et al., 2011; Pouponneau et al., 2011). Most magnetic nanoparticles for imaging agents can be prepared by oxidizing ferric ions to iron oxide aggregates at basic conditions. Different sizes of iron oxide particles have been extensively prepared to exploit their potential uses as theragnostic agents, multifunctional agents both for drug delivery vehicles and imaging agents. In order to employ magnetic nanoparticles for multifunctional purposes, many strategies are exploited to effectively incorporate drugs to magnetic nanoparticles. Surface-modifications of magnetic nanoparticles have been widely exploited to incorporate pharmaceutical drugs to the vehicles (Brzozowska and Krysinski, 2009). Doxorubicin was covalently linked to surface-hydroxyl groups of magnetic nanoparticles and confirmed the surface-decoration of doxorubicin by electrochemical methods. They successfully confirmed surface-modification of magnetic nanoparticles by various methods. Magnetite nanoparticles were surface-modified with β-cyclodextrin (β-CD) and subsequently coated Pluronic F-127. An anti-cancer drug, curcumin, was loaded into the β-CD layers via hydrophobic interactions. Loading capacity continuously increased as the amount of β-CD used for nanoparticles coating increased (Yallapu et al., 2011). Dimercaptosuccinic acid (DMSA) was coated on the surface of magnetic nanoparticles for conjugation with

* Corresponding author at: Department of Biomaterials Engineering, Kangwon National University, Chuncheon 200-701, Republic of Korea. Tel.: +82 33 250 6563; fax: +82 33 253 6560.
E-mail address: hsyoo@kangwon.ac.kr (H.S. Yoo).
[1] These authors contributed equally to this work.

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.12.044

interferon-γ (IFN-γ) which is an anti-tumorigenic cytokine. IFN-γ was adsorbed on DMSA-coated magnetic nanoparticles with a conjugation efficiency of 90% (Mejías et al., 2011). In another study, therapeutic magnetic nanoparticles were prepared by co-emulsification of doxorubicin and FeCo nanoparticles (Pouponneau et al., 2011). MR imaging was successfully performed for livers when the nanoparticles were intra-arterially injected through catheters. However, payloads of doxorubicin in the nanoparticles were not high enough to achieve therapeutic index for cancer treatments. Moreover, lacks of optimized release profiles from the vehicles were challenging issues to be overcome to obtain drug efficacies.

Pluronic, a triblock copolymer composed of poly(ethyleneoxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) (PEO-PPO-PEO), was widely employed to chemically decorate biomedical materials with the macromolecular surfactant for bio-functionalization. Pluronic and their derivatives show structural transitions between relaxed and collapsed states according to temperature changes across their lower critical solution temperatures (LCST). The thermo-responsive behaviors of Pluronic were hypothesized to be caused by entropically driven hydrophobic interactions between PPO blocks of Pluronic chains. Recently, we reported that Pluronic-decorated nanogels for gene delivery showed temperature-sensitive volume transitions, variable cytotoxicities, and distinctive transfection efficiencies according to temperature changes (Lee and Yoo, 2008). Polycation/DNA complex composed of poly(ethyleneimine) [PEI] was surface-modified with Pluronic polymers. These complexes showed variable volume changes at alternating temperatures between 4 °C and 37 °C. In a similar study, Pluronic micelles were chemically conjugated to gold nanoparticles and volume transition behaviors of the composite were extensively investigated (Bae et al., 2006). The shell cross-linked gold–Pluronic micelles exhibited volume transitions according to temperature changes. In another study, shell-cross-linked Pluronic/PEI nanocapsules were prepared via conjugation between primary amine groups of PEI and Pluronic F-127 activated by 4-nitrophenylchloroformate (Choi et al., 2006a). These nanocapsules exhibited thermal-reversible swelling/de-swelling over a temperature range of 24–33 °C because of temperature-dependent hydrophobic interaction of cross-linked and/or grafted Pluronic polymer. In another study, temperature-sensitive hyaluronic acid (HA) hydrogels were synthesized by photopolymerization of vinyl group modified HA with di-acryloyl Pluronic F-127. HA/Pluronic hydrogels showed reversible cyclic swelling and de-swelling of the hydrogels were induced by cycling temperatures between 13 °C and 40 °C in a step-wise function (Kim and Park, 2002). Pluronic/heparin nanocapsules prepared by cross-linking between heparin and activated Pluronic F-127 with *p*-nitrophenylchloroformate. They exhibited a 1000-fold volume transition and a reversible swelling and de-swelling behavior between 20 °C and 37 °C (Choi et al., 2006b).

In this study, we described a method of maximizing drug payloads in magnetic nanoaggregates. A thermo-sensitive polymer-assisted incorporation strategy was investigated for fabrication of doxorubicin-loaded magnetic nanoparticles. Temperature-driven modulation of doxorubicin incorporation was determined by dynamic light scattering and loading efficiency changes were also monitored.

2. Materials and methods

2.1. Materials

Doxorubicin was a gift from Korea United Pharm. Inc. (Seoul, South Korea). Pluronic F-127 was donated from BASF (Mount Olive, NJ). Pyridine and dichloromethane (CH_2Cl_2) were purchased from Daejung Chemicals and Metals (Shiheung, South Korea). 4-Nitrophenyl chloroformate (4-NPC), 2-iminothiolane hydrochloride (2-iminothiolane·HCl), and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO). Ethylene diamine, iron(II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$) and iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) were purchased from Junsei Chemicals (Tokyo, Japan). A549 cell line was obtained from Korea Cell Line Bank (Seoul, South Korea). RPMI1640 medium, penicillin, streptomycin, and fetal bovine serum (FBS), were purchased from Invitrogen (Carlsbad, CA).

2.2. Preparation of thiolated Pluronic F-127

The terminal hydroxyl groups of Pluronic F-127 (2.5 g) were activated by 4-NPC (0.125 g) dissolved in dichloromethane (10 ml) at room temperature for 24 h. Pyridine (0.065 g) and ethylene diamine (0.05 g) in dichloromethane (1 ml) were slowly added to the activated Pluronic and the amination reaction was performed at room temperature for 24 h (molar ratio of Pluronic:pyridine:4-NPC = 1:4:3). The amine-terminated Pluronic (2.5 g) was subsequently thiolated with 2-iminothiolane·HCl (0.28 g) in DW (10 ml) at room temperature (molar ratio of amine terminated Pluronic:2-iminothiolane·HCl = 1:10). After 12 h, the resultant solution was dialyzed against DW three times (Spectrapor6, MW cutoff = 1000) and freeze-dried.

2.3. Preparation of magnetic nanoparticles

Magnetic nanoparticles (MNP) were prepared by oxidizing ferric ions to iron oxide (magnetite) in basic conditions as described in the literatures (Yallapu et al., 2011; Banerjee and Chen, 2007). Briefly, $FeCl_3{\cdot}6H_2O$ (540 mg) and $FeCl_2{\cdot}4H_2O$ (198 mg) were completely dissolved in DW (100 ml)purged with nitrogen gas at 80 °C (molar ratio of $FeCl_3{\cdot}6H_2O$:$FeCl_2\ 4H_2O$ = 2:1). Ammonia solution (8 ml) was slowly dropped to the iron chloride solution. The reaction was performed with stirring at 80 °C for 2 h. Magnetic nanoparticles were harvested by centrifugation at 3000 rpm for 30 min and washed with DW three times.

2.4. Decoration of magnetite with thiolated Pluronic

MNP (100 mg) in dichloromethane (20 ml) was slowly dropped into thiolated Pluronic (52 mg) in dichloromethane (20 ml) and the reaction mixture was stirred for 2 h (molar ratio of Fe:thiolated Pluronic = 1:1.00, 1:0.01, and 1:0.00; MNP1.00, MNP0.01, and MNP0.00, respectively). After 3 h, Pluronic-conjugated MNP was retrieved from the reaction by magnetic forces and washed with DW three times to remove unreacted Pluronic. The morphology of Pluronic-decorated MNP was characterized by transmission energy microscopy (TEM) (Hitachi, S-4300, Japan) at the core laboratory of Kangwon National University. Briefly, Pluronic-conjugated MNPs were mounted on copper grids by dropping a solution with MNPs at 4 °C or 37 °C, which was followed by vacuum-drying at 4 °C or 37 °C for 24 h. The amount of chemically incorporated Pluronic on MNP was quantified by determining the atomic mass of carbons in the same amount of magnetic nanoparticles employing an elemental analysis system (CE Instruments, Flash 2000, UK). The amount of the incorporated Pluronic was calculated by comparing peak areas of carbon atom in elemental analysis results with respect to MNP. The peak area of carbon atom from MNP without Pluronic (MNP0.00) was set as a base line.

2.5. Self-assembly doxorubicin MNPs by thermo-responsive manners

Doxorubicin was incorporated in MNPs by thermo-sensitive manners at the elevated temperature. Pluronic decorated magnetite (30 mg) and doxorubicin (6 mg) co-dissolved in a mixture of acetone and methanol (1:1, 6 ml) were slowly dropped into pre-cooled DW (4 °C) in the presence of triethylamine (TEA) (molar ratio of doxorubicin:TEA = 1:3). After the organic phase was evaporated in vacuum, the temperature of the solution was kept 4 °C or at 37 °C to evaluate effects of temperature changes on association behaviors of the MNPs and doxorubicin incorporation. The multimodal size distribution of the MNPs at different temperatures was determined by dynamic laser light scattering equipped with a thermo-stat system (Brookhaven Instruments Corp., 90plus, USA). The amount of incorporated doxorubicin was determined by measuring the absorbance of the supernatant at 480 nm after harvesting MNPs by centrifugation.

2.6. Release of doxorubicin from MNPs

Doxorubicin release profiles from MNPs was examined by incubating doxorubicin-incorporated MNPs in dialysis membranes (Spectrapor6, MWCO = 3500). MNPs encapsulating doxorubicin (10 mg) was sealed in a dialysis bag and incubated in PBS with 1 mM or 10 mM DTT at 37 °C for 72 h. The supernatant was obtained at 0, 3, 24, 36, 72 h and the absorbance was measured by a spectrophotometer to calculate a concentration of released doxorubicin. Pre-determined concentrations of doxorubicin solutions were employed for a standard curve.

2.7. MTT-based cytotoxicity assay

Cell viability assay was assessed against A549 cells using a MTT assay as described in the literature (Park and Yoo, 2010). A549 cells were seeded in 96-well plates at cell concentration of 5×10^4 cells/ml. Cells were incubated in RPMI1640 medium containing 10% FBS and penicillin/streptomycin at 37 °C in 5% CO_2 atmosphere for 24 h. Doxorubicin-incorporated MNPs were added to each well at a final doxorubicin concentration of 0 μM to 160 μM. The same concentration of doxorubicin was employed as a control. After 48 h, the culture medium was replaced with a fresh medium and 10 μl of MTT solution (5 mg/ml) was added. After 8 h, the medium was removed and the formazan products were dissolved in a DMSO/glycine buffer for 3 h. The supernatant was harvested by magnetically retrieving MNPs and the absorbance of the supernatant was measured at 570 nm.

3. Results and discussion

Fig. 1 shows a schematic diagram of preparing MNPs encapsulating doxorubicin by a temperature-responsive manner. A hydroxyl group-terminated Pluronic was aminated by attaching ethylenediamine to the 4-NPC-activated Pluronic. The amine groups were subsequently thiolated by 2-imminothiolane to prepare thiolated Pluronic. The thiolated Pluronic was decorated on the surface-ferric oxide groups of MNPs at various molar ratios between Pluronic and Fe_3O_4. We speculated that ferric oxide groups at the surface react with the thiol groups of Pluronic, a molar ratio of 1:1 would be the maximum value that can be theoretically obtainable ratios when all ferric oxide might participate in the reaction (Table 1). The molar ratio of 1:0.01 was also employed to compare effects of Pluronic decoration on thermally inducible doxorubicin incorporation to the MNPs with respect to non-modified MNPs. In fact, many researchers employed thiol groups to surface-decorate magnetic nanoparticles composed of ferric oxide groups (Neouze and Schubert, 2008; Gooding et al., 2003). Due to the Pluronic-decorated nature of MNPs, we hypothesized that the MNP can be anchored each other at the elevated temperature by hydrophobized PPO blocks at the elevated temperature. During the association, hydrophobic doxorubicin, with an assistance of triethylamine, is simultaneously co-encapsulated in MNP aggregates to form doxorubicin-encapsulated MNPs. Furthermore, the linkage between iron oxide and thiol groups can be cleaved in response to redox potential and the decorated Pluronic can be subsequently liberated.

Table 1
Determination the amount of the immobilized Pluronic on MNPs by the elemental analysis of Pluronic-decorated MNPs.

MNPs	Feed ratio of Pluronic to Fe_3O_4[a] (%, mol/mol)	Elemental weight of carbon in MNPs[b] (%, w/w)	Immobilized Pluronic on Fe_3O_4[c] (%, mol/mol)
MNP1.00	1.00	6.99	0.23
MNP0.01	0.01	2.51	0.06
MNP0.00	0.00	1.07	–

[a] The molar ratio of the thiolated Pluronic to Fe_3O_4.
[b] Peak areas of C in the elemental analysis spectrum.
[c] The amount of Pluronic was calculated based on the peaks area of carbon in the elemental analysis results.

The amount of the conjugated Pluronic was confirmed by the elemental analysis of Pluronic-decorated MNPs as shown in Table 1. The amount of the decorated Pluronic was calculated based on the amount of C atoms with respect to the amount of total weight of MNPs employed for the analysis. When the initial ratios of Pluronic to Fe_3O_4 were 1.00, 0.01, and 0.00 (MNPs without Pluronic), the actual amounts of immobilized Pluronic moieties were 0.23, 0.06, and 0 mole per one mole of Fe_3O_4. This result clearly suggests that Pluronic chains were successfully tethered to the surface of MNPs in accordance with the feed ratio of Pluronic to Fe_3O_4. Thus, we expected that the amount of encapsulated doxorubicin in MNPs was dependent on the surface density of Pluronic moieties because of the thermo-responsibility of MNP would be also expected to increase as well.

MNPs with different numbers of Pluronic moieties were characterized for their morphological features by TEM as shown in Fig. 2. In order to obtain TEM images according to the temperatures, we vacuum-dried MNPs at the designated temperatures at 4 °C and 37 °C as described in Section 2. At 37 °C, no difference was observed among MNPs irrespective of Pluronic decoration. However, at 4 °C, MNP1.00 showed distinct clouds of polymeric shell around a bunch of MNPs; however, no cloud of the polymeric shells was clearly visualized in MNPs at those with lower decoration degrees such as MNP0.01 or MNP0.00. This could be attributed to the thermo-responsive structural changes of Pluronic moieties on MNPs. At lower temperatures, Pluronic is fully extended to aqueous phases and this forms 'core-shell like structure' for Pluronic-decorated MNPs. However, MNPs with lower degree of decoration of Pluronic (MNP0.01) are unlikely to show the core-shell feature because of low substitution densities at the surface of MNPs. In fact, as shown in Table 1, the number of Pluronic per Fe_3O_4 was 0.23 and 0.06 for MNP1.00 and MNP0.01, respectively (mol/mol). Thus, thermo-responsive encapsulation was accordingly tested for these MNPs at both temperatures. Many researchers also observed core/shell structures of magnetic nanoparticles containing iron oxide when they surface-decorated them with hydrophilic polymers such as PEO and PEG (Yallapu et al., 2010; Ha¨feli et al., 2009).

Fig. 3 shows multimodal size distributions and proposed models of doxorubicin-encapsulated MNPs at different temperatures. MNP1.00 showed a dramatic size change when the temperature increased from 4 °C to 37 °C. At 4 °C, the size distributions of MNPs

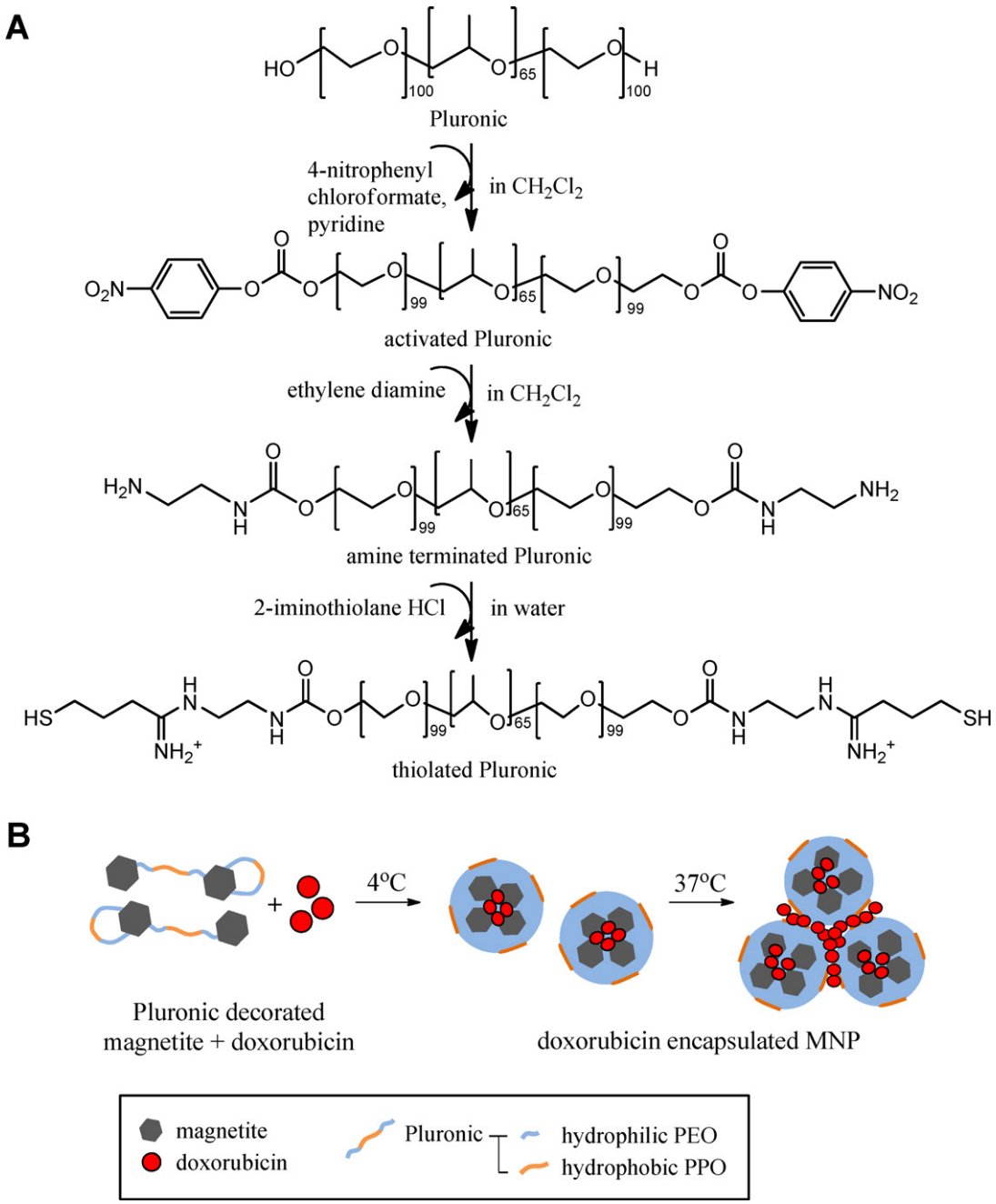

Fig. 1. Schematic diagram of preparing magnetic nanoparticle (MNP) encapsulating doxorubicin. (A) Synthesis of thiolated Pluronic and (B) thermo-responsive incorporation of doxorubicin in MNP.

showed mono-dispersed characteristics (Fig. 3A). Due to Pluronic decoration on MNPs, they are well-separated each other without any prominent aggregations. This can be attributed to stabilization of MNPs by Pluronic, a non-ionic surfactant, because Pluronic dissociates the MNPs by steric hindrance in the same way that oleate or poly(vinyl alcohol) [PVA] behaves, which are frequently employed for stabilizing magnetic particles during the preparation of magnetites (Yallapu et al., 2010; Kayal and Ramanujan, 2010). For MNP0.01 or MNPs without Pluronic (MNP0.00), however, the stabilization effects were significantly diminished in responses to the number of the Pluronic pendants (Fig, 3C and E). Specifically, MNP0.00 showed the most severe aggregation behaviors at 4 °C and no single particles of magnetite were observed as shown in Fig. 3E. However, the average size of MNPs dramatically increased to about 300 nm at 37 °C; 15 times larger diameter compared those particles at 4 °C. At 37 °C, hydrophobized PPO blocks become hydrophobic and subsequently associated each other by strong hydrophobic interactions, suggesting that MNPs with considerable degrees of Pluronic decoration also forms nano-aggregates as shown in Fig. 2. During the association process, doxorubicin is also incorporated into the pendant arms of MNPs by hydrophobic interactions. Doxorubicin–HCl, a water-soluble form of doxorubicin, can be readily hydrophobized by scavenging chloride ions by adding TEA (Yoo and Park, 2004a, b). Thus, the hydrophobized doxorubicin is incorporated into the clusters of MNP1.00 at the elevated temperature above LCST. However, MNP0.01 or MNP0.00 did not show such a dramatic size transition from the size at 4 °C. This is certainly caused by their existing aggregate of MNPs at 4 °C. MNP0.01 or MNP0.00 were already aggregated into the clusters of MNPs around several hundred nanometers in diameters as shown

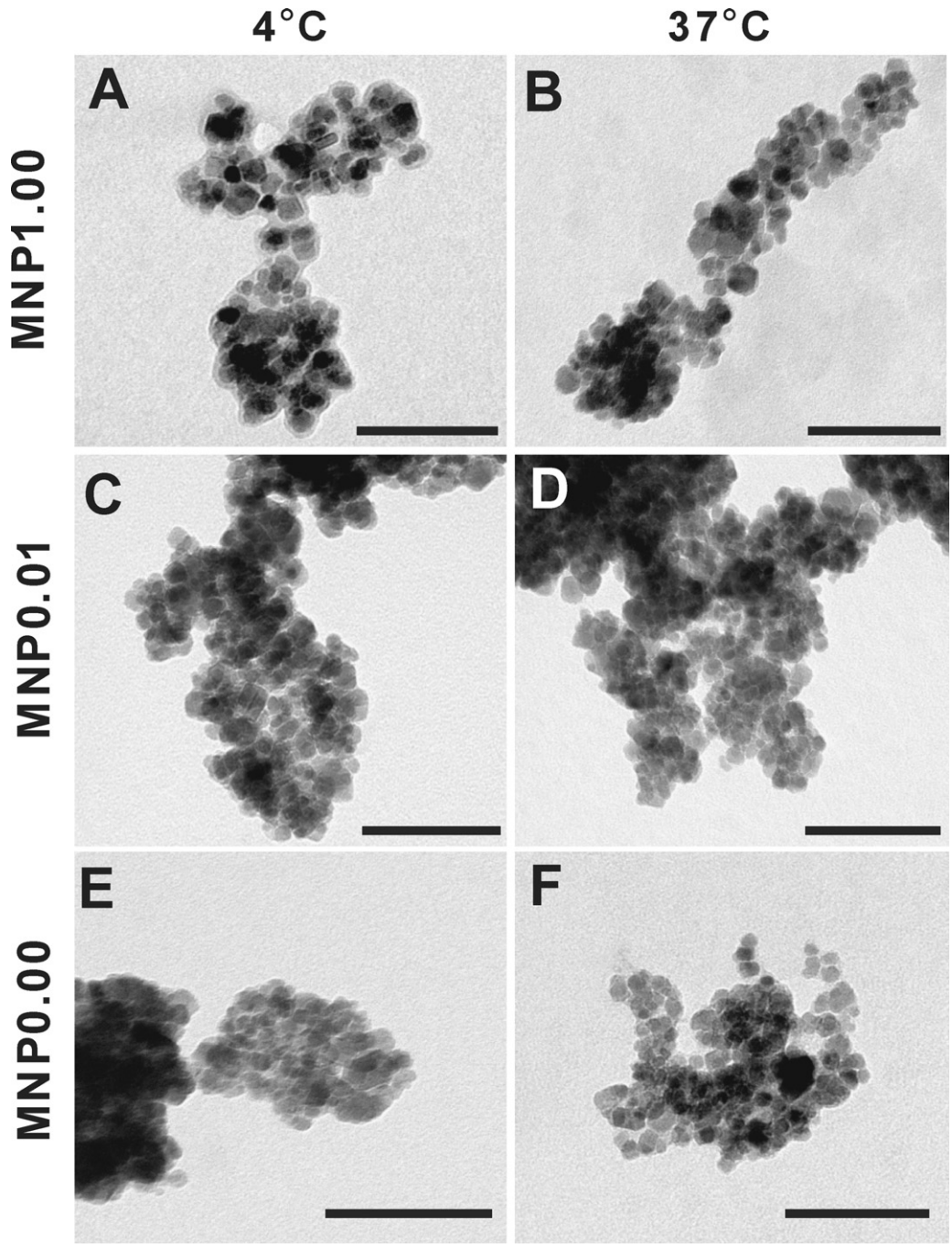

Fig. 2. Transmission electron microscopy (TEM) images of MNPs. MNPs were vacuum dried at 4 °C (A, C and E) or at 37 °C (B, D and F), respectively (scale bar = 100 nm).

in Fig. 3C and E and these behaviors subsequently decreased the thermo-responsibilities of MNPs.

Fig. 4 shows loading efficiency changes of doxorubicin in the clusters of MNPs at different temperatures. At 4 °C, the loading efficiency of doxorubicin was confirmed to be lower than 30% for all MNPs; MNP1.00 showed the highest loading efficiency over other MNPs, which was followed by MNP0.01 and MNP0.00. The incorporation behaviors of doxorubicin in MNPs can be attributed to the different hydrophobic interactions between doxorubicin and various MNPs. Because MNP1.00 showed the least clustering of MNPs due to the higher degree of Pluronic decoration, it is clear that MNP1.00 at 4 °C has the higher surface area of Fe_3O_4 compared MNP0.01 and MNP0.00 (Fig. 3A, C and E). The higher surface areas of the MNPs subsequently maximize strong hydrophobic interactions between doxorubicin and MNPs over other MNPs. When the temperature increased to 37 °C, the loading efficiency of MNPs dramatically increased for MNP1.00. However, MNP0.01 or MNP0.00 did not show any dramatic increase in loading efficiencies of doxorubicin at the elevated temperature. This result can be clearly attributed to the degree of Pluronic decoration in MNPs. The degree of the Pluronic decoration may play two critical roles on controlling loading efficiencies of doxorubicin in MNPs. First, the lower degrees of MNPs aggregations at 4 °C are more favorable toward the higher loading of doxorubicin because MNPs at a 'relaxed' state can maximize contacts with the surrounding doxorubicin. This is a critical step toward the next stage, the drug incorporation because the drug should be incorporated by the structural changes of the decorated Pluronic on the surface of MNPs. At 37 °C, the degree of

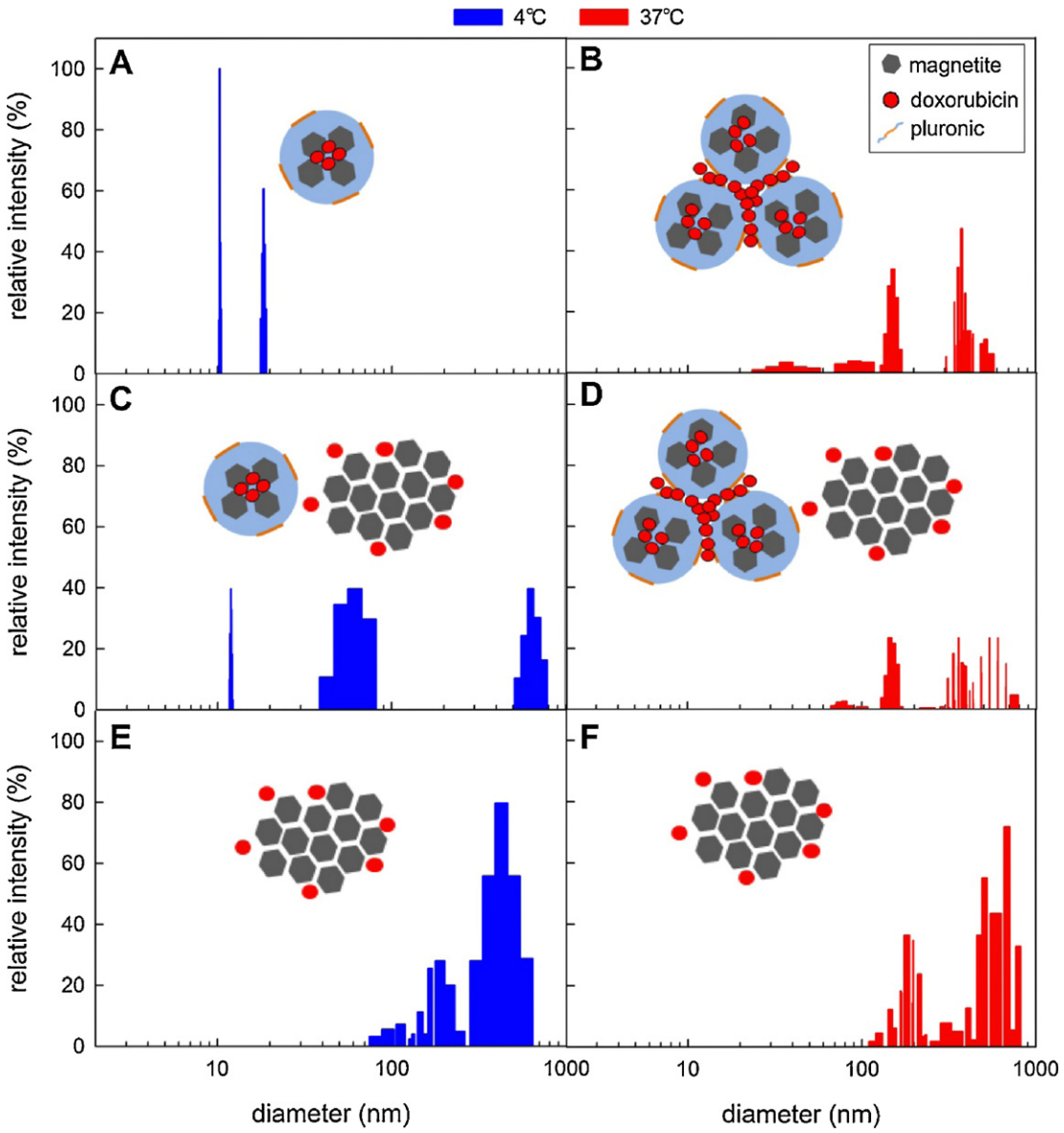

Fig. 3. Multimodal size distribution of MNP1.00 (A and B), MNP0.01 (C and D) and MNP0.00 (E and F), which was measured by dynamic light scattering (DLS) at 4 °C (A, C and E) and 37 °C (B, D and F). Average diameters of (A), (B), (C), (D), (E) and (F) were 14.38 nm, 303.10 nm, 235.99 nm, 396.89 nm, 349.53 nm and 394.12 nm, respectively.

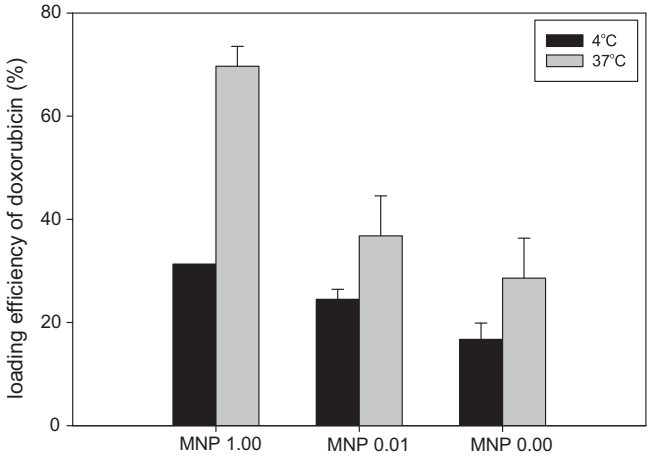

Fig. 4. Loading efficiency of doxorubicin in MNPs. Doxorubicin was encapsulated into each MNP at 4 °C or 37 °C with triethylamine and the average loading efficiency was calculated based on the initial amount of doxorubicin ($n = 3$).

Pluronic clearly affects the hydrophobic interaction between doxorubicin and MNPs. Many studies previously indicated that critical densities of polymeric decoration on surfaces are required for the modulated functionality of the modified surfaces. Thus, we speculate that MNP1.00 has the critical number of Pluronic decoration on the surface for thermo-sensitive drug loading because MNP0.01 did not show any dramatic change of drug loading efficiencies.

In order to evaluate drug release profiles from doxorubicin incorporated MNPs, the MNPs were exposed to different degree of reducing conditions by changing DTT levels in the incubation medium (Fig. 5). We speculated that the decorated Pluronic on the MNPs can be liberated from the MNPs by cleaving the linkages between Fe_3O_4 and Pluronic, subsequently accelerating release rates of doxorubicin from the MNPs. The release rates did not change until the concentration of DTT was increased to 10 mM irrespective of the amount of decorated Pluronic on the MNPs. In PBS or 1 mM DTT solution, the amount of released doxorubicin was below 20% for 72 h and no difference was found among the MNPs. In 10 mM DTT solutions, however, the amount was significantly increased to 65%–82% according to the amount of decorated

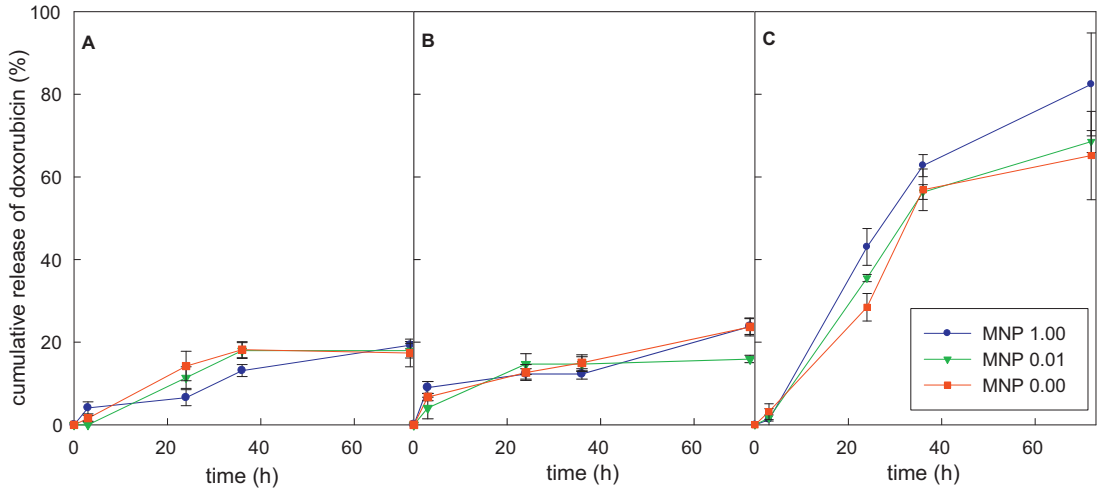

Fig. 5. Release profiles of doxorubicin from DOX-MNPs. DOX-MNPs were incubated in PBS (A), with 1 mM DTT (B), and 10 mM DTT (C) at 37 °C.

Pluronic moieties on the MNPs. Thus, this result clearly suggests that the linkages between Pluronic and MNPs can be cleaved in response to the redox potential of the solutions and the incorporated doxorubicin is released accordingly due to the liberation of Pluronic moieties from the MNPs. Many studies employing Au–thiol linkages or Fe_3O_4–thiol linkages indicated that those linkages are cleaved at a DTT concentration range of 1 mM–10 mM (Kim et al., 2006; Sun et al., 2010). However, in comparison with other studies employing a direct conjugation between drug and carriers, we did not chemically conjugate doxorubicin to MNPs, but tethered doxorubicin to the carriers by thermo-responsive manners. Several advantages are featuring this strategy over conventional methods employing chemical conjugation of drugs. The incorporated drugs were not chemically modified and the native biological activities of the incorporated drugs, therefore, are not changed. Additionally, a complex chemistry for drug conjugation can be avoided to maximize drug payload in the carriers.

Cytotoxicity of doxorubicin-incorporated MNPs was tested against A549 cell lines as shown in Fig. 6. In comparison to intact doxorubicin, doxorubicin-incorporated MNPs showed slightly inferior cytotoxicities to doxorubicin; no difference was found among MNPs with different degrees of Pluronic decoration. Thus, we speculated that all doxorubicin was not liberated from the aggregates of MNPs due to hydrophobic interactions. This result can be supported by the doxorubicin release profiles shown in Fig. 5. In 1 mM DTT or PBS, only 20% of the incorporated doxorubicin was released from the MNPs for 3days, suggesting that drug efficacy can be attenuated although the same amount of doxorubicin equivalents was initially added to the cells. Because nanoparticles engulfed by cells experience endosomal pathways from endosomes to lysosomes, doxorubicin release from the MNPs mainly rely on reducing conditions in endosomes and lysosomes. Many literatures previously determined the reducing powers of the small cytosolic organelles such as endosomes and lysosomes with various methods (Wang and Ballatori, 1998). Therefore, we concluded that doxorubicin-incorporated MNPs showed different degrees of cytotoxicities in response to cellular reducing conditions. Furthermore, we speculate that the doxorubicin can be only dissociated from the MNPs within cells, but not during the systemic circulation when those particles are intravenously administered. Thus, the doxorubicin-incorporated MNPs can be a bonafide example of therapeutic imaging agents, which can be further applied for determining real-time therapeutic efficacy of anti-cancer drugs by MR imaging.

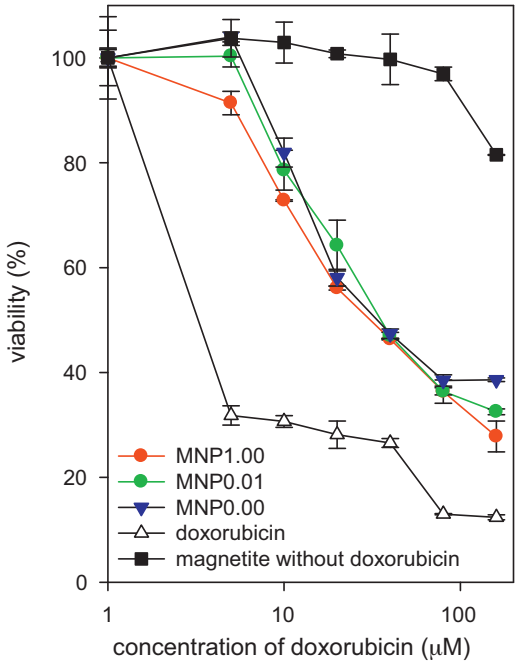

Fig. 6. Cell viability against A549 cells by a MTT assay. Cells were incubated in RPMI1640 with 10% fetal bovine serum at 37 °C in 5% CO_2 atmosphere for 48 h with various concentrations of doxorubicin incorporated MNPs.

4. Conclusion

Thiolated Pluronic was decorated on surface of MNPs and incorporation of doxorubicin was dependent on the degree of Pluronic decoration. The incorporated doxorubicin was released in response to reducing conditions of the release medium. Doxorubicin-loaded MNPs showed attenuated cytotoxicities against A549 cells compared to native doxorubicin.

Acknowledgment

This study was supported by a grant from the National R&D Program for Cancer Control, Ministry for Health and Welfare, Republic of Korea (1020420).

References

Bae, K.H., Choi, S.H., Park, S.Y., Lee, Y., Park, T.G., 2006. Thermosensitive Pluronic micelles stabilized by shell cross-linking with gold nanoparticles. Langmuir 22, 6380–6384.

Banerjee, S.S., Chen, D., 2007. Magnetic nanoparticles grafted with cyclodextrin for hydrophobic drug delivery. Chem. Mater. 19, 6345–6349.

Brzozowska, M., Krysinski, P., 2009. Synthesis and functionalization of magnetic nanoparticles with covalently bound electroactive compound doxorubicin. Electrochim. Acta 54, 5065–5070.

Choi, S.H., Lee, S.H., Park, T.G., 2006a. Temperature-sensitive Pluronic/poly(ethylenimine) nanocapsules for thermally triggered disruption of intracellular endosomal compartment. Biomacromolecules 6, 1864–1870.

Choi, S.H., Lee, J., Choi, S., Park, T.G., 2006b. Thermally reversible Pluronic/heparin nanocapsulesexhibiting1000-fold volume transition. Langmuir 22, 1758–1762.

Gooding, J.J., Mearns, F., Yang, W., Liu, J., 2003. Self-Assembled monolayers into the 21st century: recent advances and applications. Electroanalysis 15, 81–96.

Ha¨feli, U.O., Riffle, J.S., Shekhawat, L.H., Baranauskas, A.C., Mark, F., Dailey, J.P., Bardenstein, D., 2009. Cell uptake and *in vitro* toxicity of magnetic nanoparticles suitable for drug delivery. Mol. Pharmaceut. 6, 1417–1428.

Kayal, S., Ramanujan, R.V., 2010. Doxorubicin loaded PVA coated iron oxide nanoparticles for targeted drug delivery. Mater. Sci. Eng. 30, 484–490.

Kim, M.R., Park, T.G., 2002. Temperature-responsive and degradable hyaluronic acid/Pluronic composite hydrogels for controlled release of human growth hormone. J. Control. Release 80, 69–77.

Kim, S.H., Jeong, J.H., Lee, S.H., Kim, S.W., Park, T.G., 2006. PEG conjugated VEGF siRNA for anti-angiogenic gene therapy. J. Control. Release 116, 123–129.

Lee, J.I., Yoo, H.S., 2008. Pluronic decorated-nanogels with temperature-responsive volume transitions, cytotoxicities, and transfection efficiencies. Eur. J. Pharm. Biopharm. 70, 506–513.

Mejías, R., Pérez-Yagüe, S., Gutiérrez, L., Cabrera, L.I., Spada, R., Acedo, P., Serna, C.J., Lázaro, F.J., Villanueva, Á., Morales, M.P., Barbera, D.F., 2011. Dimercaptosuccinic acid-coated magnetite nanoparticles for magnetically guided in vivo delivery of interferon gamma for cancer immunotherapy. Biomaterials 32, 2938–2952.

Neouze, M., Schubert, U., 2008. Surface modification and functionalization of metal and metal oxide nanoparticles by organic ligands. Monatsh. Chem. 139, 183–195.

Park, S., Yoo, H.S., 2010. In vivo and in vitro anti-cancer activities and enhanced cellular uptakes of EGF fragment decorated doxorubicin nano-aggregates. Int. J. Pharm. 383, 178–185.

Pouponneau, P., Leroux, J., Soulez, G., Gaboury, L., Martel, S., 2011. Co-encapsulation of magnetic nanoparticles and doxorubicin into biodegradable microcarriers for deep tissue targeting by vascular MRI navigation. Biomaterials 32, 3481–3486.

Sun, H., Guo, B., Li, X., Cheng, R., Meng, F., Liu, H., Zhong, Z., 2010. Shell-sheddable micelles based on dextran-SS-poly(ε-caprolactone) diblock copolymer for efficient intracellular release of doxorubicin. Biomacromolecules 11, 848–854.

Wang, W., Ballatori, N., 1998. Endogenous glutathione conjugates: occurrence and biological functions. Pharmacol. Rev. 50, 335–356.

Yallapu, M.M., Othman, S.F., Curtis, E.T., Gupta, B.K., Jaggi, M., Chauhan, S.C., 2011. Multi-functional magnetic nanoparticles for magnetic resonance imaging and cancer therapy. Biomaterials 32, 1890–1905.

Yallapu, M.M., Foy, S.P., Jain, T.K., Labhasetwar, V., 2010. PEG-functionalized magnetic nanoparticles for drug delivery and magnetic resonance imaging applications. Pharm. Res. 27, 2283–2295.

Yoo, H.S., Park, T.G., 2004a. Folate receptor targeted biodegradable polymeric doxorubicin micelles. J. Control. Release 96, 273–283.

Yoo, H.S., Park, T.G., 2004b. Folate-receptor-targeted delivery of doxorubicin nano-aggregates stabilized by doxorubicin–PEG–folate conjugate. J. Control. Release 100, 247–256.