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Cytotoxicity of release products from magnetic nanocomposites in targeted drug delivery

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Abstract

The efficacy of chemotherapy can be significantly improved if the therapeutic agent remains localized at the afflicted area and released at controlled rates. Such a targeted drug delivery can be achieved using magnetic nanocomposite (MNC), which incorporates drug and magnetic nanoparticles in biodegradable polymer microspheres. Reported here are results from an *in vitro* study on drug release rate and cytotoxicity of other release products from MNC. The model system contains an anti-cancer chemotherapy agent 5-flurouracil (5-FU) and $(Co_{0.5}Zn_{0.5})Fe_2O_4$ in poly(lactic-co-glycolic acid) (PLGA) matrix produced by an oil/oil emulsion technique. Cell proliferation data indicate a sustained release of 5-FU for mouse macrophage cell eradication, whereas other microsphere components of magnetic nanoparticles and PLGA have little cytotoxic effects.

Keywords

targeted drug delivery, magnetic nanocomposites, drug release rate, cytotoxicity

Introduction

Nanotechnology has been advancing rapidly in recent years. It now prevails in many crucial areas of application, ranging from chemical catalysis to semiconducting and optoelectronic devices, environment-friendly energy systems, and beyond. One of the most promising applications focuses on the development of magnetic nanocomposite (MNC) as carriers for site-specific delivery of drugs.[1] Relevant to such an approach are drug release rate and cytotoxicity of other release products from MNC. A model system used in this *in vitro* study is based on micron-size MNC spheres containing anti-cancer chemotherapy agent 5-flurouracil (5-FU)[2] and ferrite $(Co_{0.5}Zn_{0.5})Fe_2O_4$ nanoparticles (MNPs) in a biocompatible and biodegradable poly(lactic-co-glycolic acid) (PLGA) matrix. Superparamagnetic behaviors of single-domain ferrite nanoparticles[3,4] yield relatively high saturation magnetization suitable for MNC localization under an applied magnetic field. On the other hand, at body temperatures above the superparamagnetic blocking temperature, they have no magnetic remanence, thus avoiding MNC agglomeration and the subsequent blood clotting after the applied field is removed.

The drug release from MNC occurs through three mechanisms: swelling and diffusion, diffusion, and degradation. Hydration of the polymeric spheres leads to swelling, yielding an increased free volume to allow a 'burst' of release products. Diffusion is the most desired mechanism because it provides a nearly constant drug release rate. Following the diffusion stage, the polymeric MNC starts to degrade with increasing rate of release.[5]

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Experimental

Magnetic nanoparticle preparation

Mixed-MNPs were prepared by co-precipitation of $CoSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and $Fe_2(SO_4)_3 \cdot 7H_2O$. The sulfates were mixed in stoichiometric ratios in distilled water, with constant stirring by a magnetic bar at 700 rpm for 3 h at 80°C. NaOH solution was then added drop-wise as a precipitant. A dark green suspension formed gradually, at pH = 12.5. After settlement, the precipitates were washed 10 times to remove the excess sodium sulfate. Meanwhile, pH decreased from 12.5 to 7.5. The final product, after being dried at room temperature, was confirmed to be $(Co_{0.5}Zn_{0.5})Fe_2O_4$ by the X-ray diffraction pattern shown in Figure 1. Meanwhile, the mean particle size of approximately 10 nm is derived from the (3 1 1) peak and the Scherrer's formula.[6]

Microsphere fabrication

In the nanocomposite sphere fabrication process, two dissimilar oil phases were prepared. For the *first oil phase*, PLGA (mw 40,000–75,000) was added to acetonitrile as solvent and placed in a covered conical flask having a magnetic stirring bar. The mixture was kept on a hotplate for 30 min to dissolve the PLGA completely. At selected weight ratios, 5-FU and ferrite nanoparticles were added and well dispersed. The *second oil phase* was prepared by adding Span 80 as a surfactant to a heavy liquid of paraffin oil. The mixture was placed under an overhead mixer operated at 7000 rpm with a specially designed high-shear, sharp impeller.[7]

The *first oil phase* was then added drop-wise to the *second oil phase*. The mixer was kept running for 1.5 h to evaporate acetonitrile. MNC spheres in the viscous oil were collected by centrifugation at 17,000 rpm for 30 min at 10°C and washed with *n*-hexane and carbon tetrachloride to completely remove the heavy paraffin oil. The final product (200 nm–1.5 μm) was filtered through a 200-nm media under a 25-in. Hg vacuum, washed three times with deionized water at 4°C and dried. Superparamagnetic behaviors of the $(Co_{0.5}Zn_{0.5})Fe_2O_4$ nanoparticles inclusion are clearly shown in Figure 2(a), where the peak temperature near 130 K for zero-field-cooling magnetization corresponds to the blocking temperature T_B of single-domain nanoparticles. As expected, a reversible $M(H)$ curve at $300\,K > T_B$ in Figure 2(b) provides a saturation magnetization of approximately 2500 Oe.[8]

Three samples used in this study are listed in Table 1. Sample A contains 5-FU and magnetic nanoparticles in PLGA. Sample B contains magnetic nanoparticles but no 5-FU in PLGA. Sample C has PLGA as the sole ingredient.

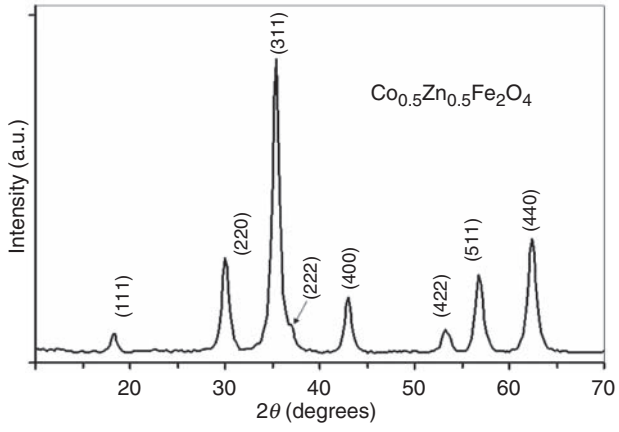

Figure 1. X-ray diffraction patterns of $Co_{0.5}Zn_{0.5}Fe_2O_4$ nanoparticles.

Cell culture and cytotoxicity assay

Mouse macrophage cell line Raw 264.7 cells (ATCC #TIB-71) were cultured overnight in Dulbecco's modified Eagle's medium (ATCC #30-2002) with 10% fetal bovine serum (ATCC #3020) on 96-well plates (5000 cells/well/100 μL) inside an incubator (37°C, 5% CO_2 in air). The next day, 100 μL of Samples A, B, and C at a concentration of 10 mg/mL were added into the wells of first column to mix with the 100 μL of the culture medium, followed by serial dilution to transfer 100 μL of the mixture to the subsequent wells in each column. The final MNC content per well was diluted by half resulting in a weight addition from 0.25 to 500 μg. Wells without MNC co-cultures were seeded with cells as a positive control. In addition, different amounts of pure 5-FU in medium without MNC were included into wells seeded with cells to demonstrate the response of cells to the chemotherapy. The culture plates were kept in the incubator for different periods of time before 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Drug release test

The functional drug release pattern of the 5-FU-containing microspheres was examined on Raw cells. Ten milligrams of 5-FU-containing magnetic microspheres were dissolved in 1 mL of culture medium in a sterile culture tube for 24 h at 37°C before harvesting the supernatant (1 mL) for Day 1 Fluorouracil release. Same amount (1 mL) of fresh medium was added back to the tube, and the same procedures were repeated after 3, 5, and 7 days, yielding Days 3, 5, and 7 drug release media.

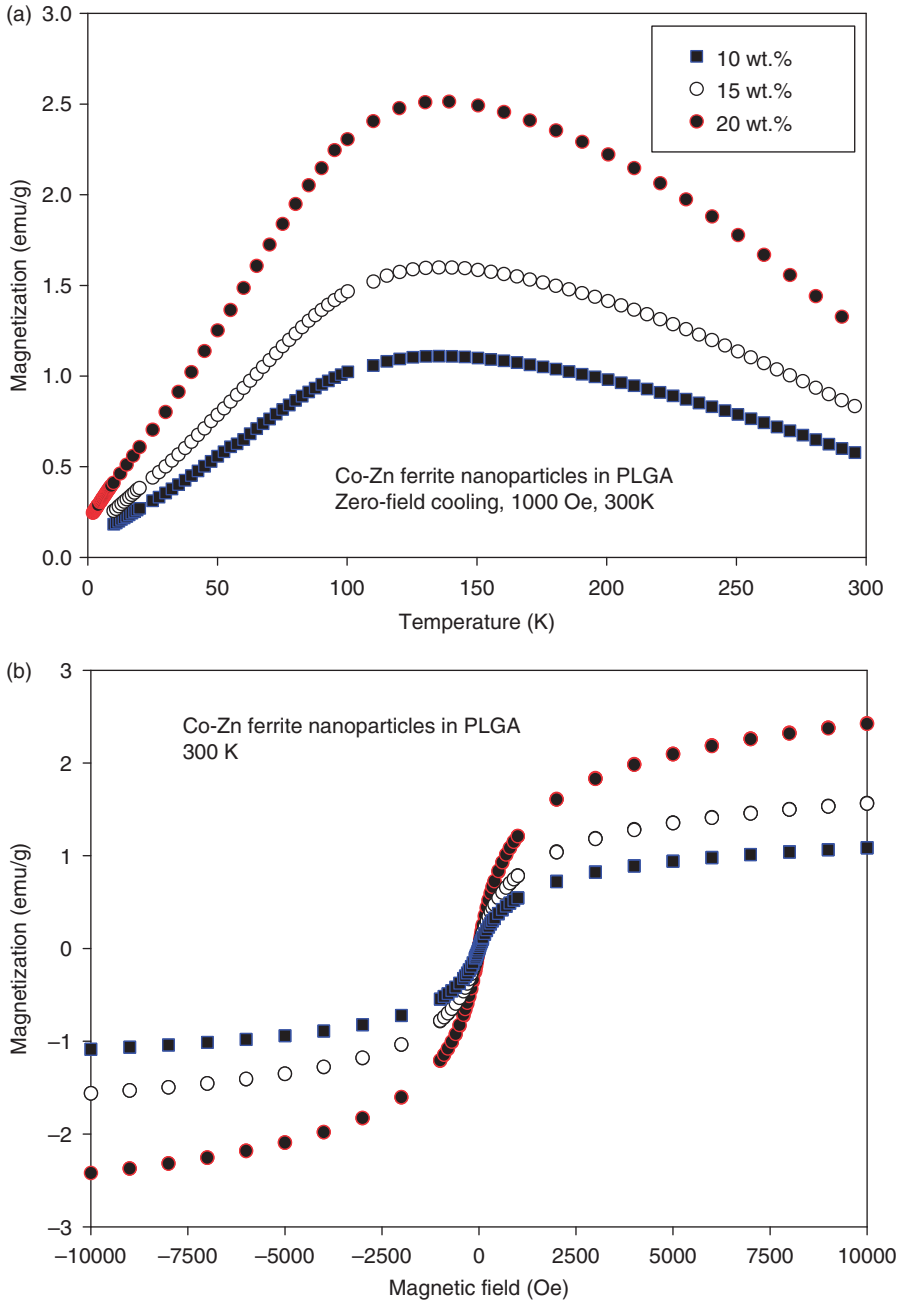

Figure 2. (a) Temperature dependence of magnetization and (b) magnetic hysteresis curves of MNP in PLGA.

Table 1. List of MNC samples with different ingredients

Sample	5-FU (%)	MNP	PLGA (%)
A	20	10% $Co_{0.5}Zn_{0.5}Fe_2O_4$	70
B	0	10% $Co_{0.5}Zn_{0.5}Fe_2O_4$	90
C	0	0%	100

Raw cells were seeded in a 96-well culture plate at 2×10^5 per well for 24 h before introduction of the 5-FU release supernatant samples. Each individual drug release supernatant sample was tittered, according to the original sphere concentration, from 500 to 1 µg/well. Cells were then incubated at 37°C in 5% CO_2 atmosphere for 6 days, followed by the MTT assay.

MTT proliferation assay

Standard MTT-based tests were employed to assess cell survival and proliferation. The yellow tetrazole MTT is reduced to purple formazan by the reductase enzymes in living cells.[9] These reductions take place only when

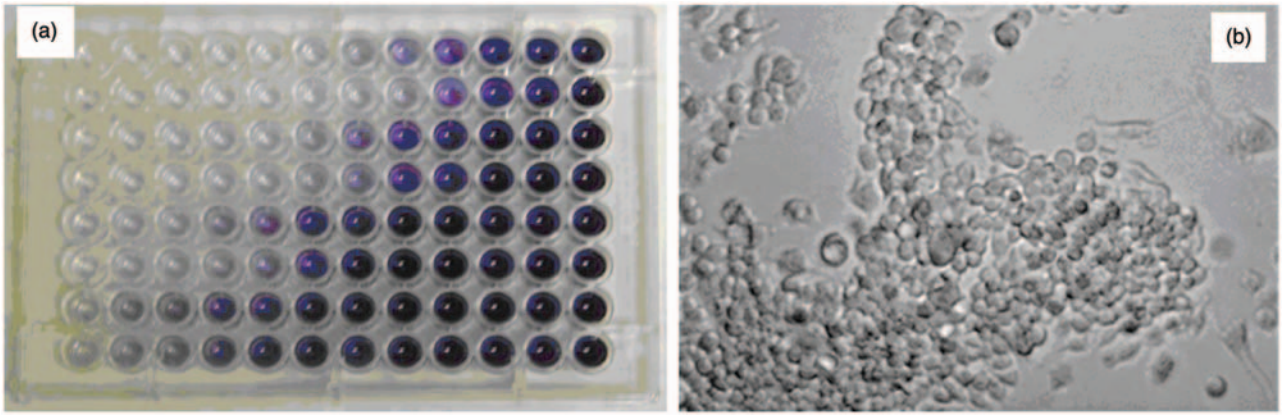

Figure 3. (a) Visual observation of microplate wells showing darker color for more viable cells and (b) microscopic image of macrophage cells after 3 days in culture.

reductase enzymes are active. After a certain number of days of culture, 20 μL of MTT solution was added to each well of the culture plate. The plate was kept for 6 h at 37°C before the addition of 10% sodium dodecyl sulfate. The cell culture plate was then placed in the incubator overnight. The purple formazan product is dissolved in, for example, dimethyl sulfoxide, to form a colored solution, as shown in Figure 3(a). The mitochondrial activity of the cells was then determined through the absorbance of this colored solution by measuring its optical density (OD) at a 590 nm wavelength on a microplate photospectrometer (Molecular Devices, Menlo Park, CA). The cell viability can be calculated by normalizing the OD reading of the control to 100% cell viability.

Results and discussion

Six different samples were made to quantify and compare the magnetic properties and biocompatibility in drug delivery systems. MNCs were developed containing 10%, 15%, and 20% cobalt zinc ferrite with the remaining PLGA. Tests were conducted to characterize the magnetization values of the samples. Twenty percent cobalt zinc ferrite nanoparticles were selected for further biocompatibility tests due to the higher magnetization value. Three MNCs were produced to quantify the contributing cytotoxicity of the materials used in the MNC. Sample A contained 20% 5-Fu, 10% cobalt zinc ferrite, and 70% PLGA. Sample B contained 0% 5-Fu, 10% cobalt zinc ferrite, and 90% PLGA. Sample C contained 0% 5-Fu, 0% cobalt zinc ferrite, and 100% PLGA. A negative control was included in the study of the 100% 5-Fu.

Figure 3(b) shows a microscopic appearance of cultured macrophage cells at Day 3 cultivation without MNC challenges. The Raw cells had normal shape and size with no noticeable deformation or death. Cell viability among different treatment groups was evaluated by normalized MTT assay and expressed as the percentage of cell survival after certain days of treatment, overdoses of therapeutic drug (5-FU), or concentrations of drug delivery spheres. Figure 4(a) illustrates the cell survival on the positive control group (5-FU addition to cells). Days 1, 3, and 5 all produce approximately the same curve with the exception of Day 1 which levels off at 20% cell viability. Days 3 and 5 show a leveling-off around 6% cell viability. While many factors are involved with taking OD readings, one possible reason for cell viability leveling-off at high concentrations is that a small portion of the cells are resistant to 5-FU chemotherapy. It appeared that 20% of the cells survived in the first day of 5-FU treatment, while 6% of the resistant cells remained at Days 3 and 5 following 5-FU treatments. Since Days 3 and 5 have approximately the same cell viability, it is possible that the rate of mutation during cell division to that of 5-FU effect reaches a steady state at 6%. Concentration of significant impact of 5-FU is about 10 μg/100 μL. An expanded view of this region is illustrated in the insert of Figure 4(a). Significant reductions in cell viability were obtained from 0 to 10 μg, and then leveled off. To reduce side effects, it is desirable to keep the chemotherapeutic agent at the minimum dose with the maximum therapeutic effect.[10]

In comparison, Figure 4(b) shows the cell cytotoxicity of Sample A, which contains 20% 5-FU and 10% $(Co_{0.5}Zn_{0.5})Fe_2O_4$ in PLGA matrix. Massive cell growth inhibition (>90%) was observed in cells with MNC containing 5-FU. From the insert in Figure 4(b), the critical weight addition of Sample A is around 1%. However, the exponential increase in viability is considerably lower than the addition of pure 5-FU, especially after 6 days. Three possible reasons for the increased toxicity at lower concentrations include the materials making up of MNC (MNP and PLGA), the

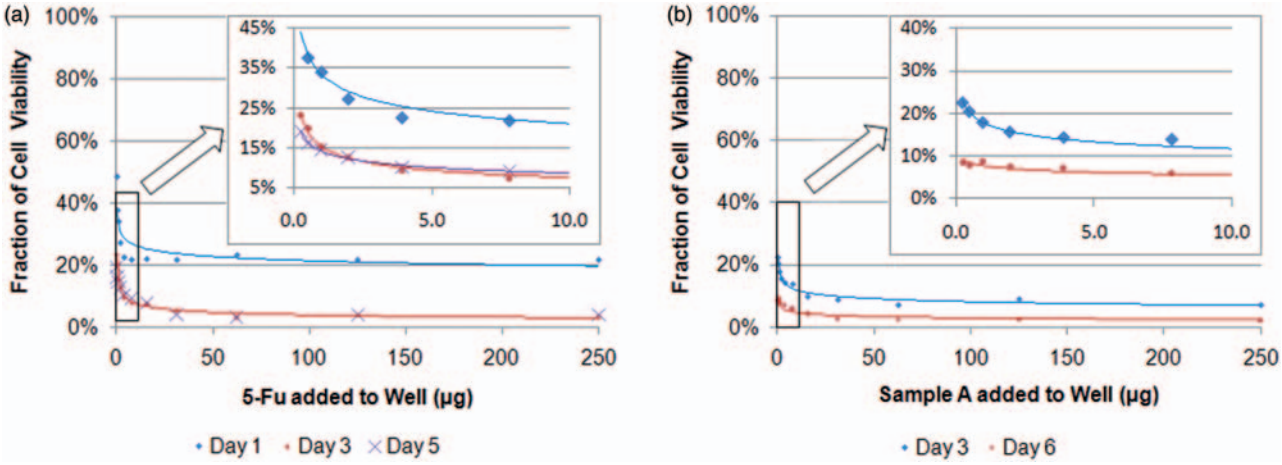

Figure 4. Inhibition of cell growth in medium containing (a) 5-FU only and (b) Sample A.

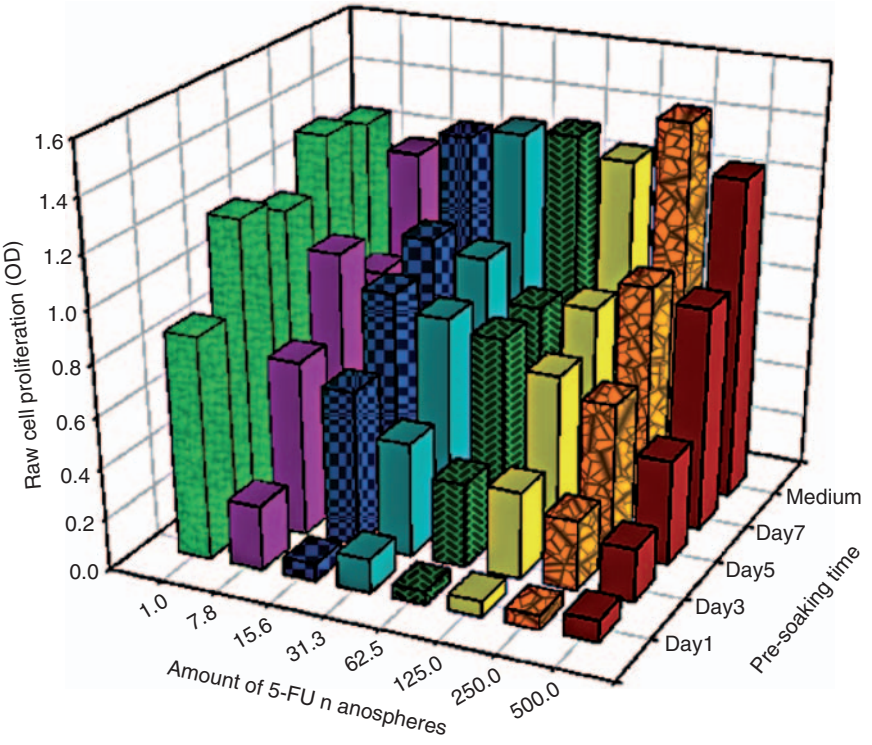

Figure 5. Drug release assay on Raw cells with 5-FU nanospheres.

MNC susceptible to be engulfed,[11] and the controlled release rates inherent to the PLGA polymer-based drug delivery systems.[12,13] It is generally thought that magnetic nanospheres are taken up by endocytosis, which then release the therapeutic agent at intracellular locations. Even during the extracellular stage, the controlled release rate maintains sustained drug concentration that creates a harsher environment for tumor cells to survive. In the current drug release study, the chemo-agent 5-FU had a burst release from the composite spheres to initiate therapeutic effects, and it maintained cell-killing ability over a relative long period of time up to 7 days at most tested concentrations of the composite spheres (Figure 5).

Further study was performed to evaluate the toxicity of the biomaterial components in the drug delivery nanocomposites. Raw cells were cultured in the presence of MNC that did not contain the chemotherapy agent (Sample B, Figure 6(a)). MTT assay suggested a significant increase in cell viability with empty MNC co-cultures, compared to that with Sample A. However, Figure 6(a) shows some cytotoxicity of Sample B,

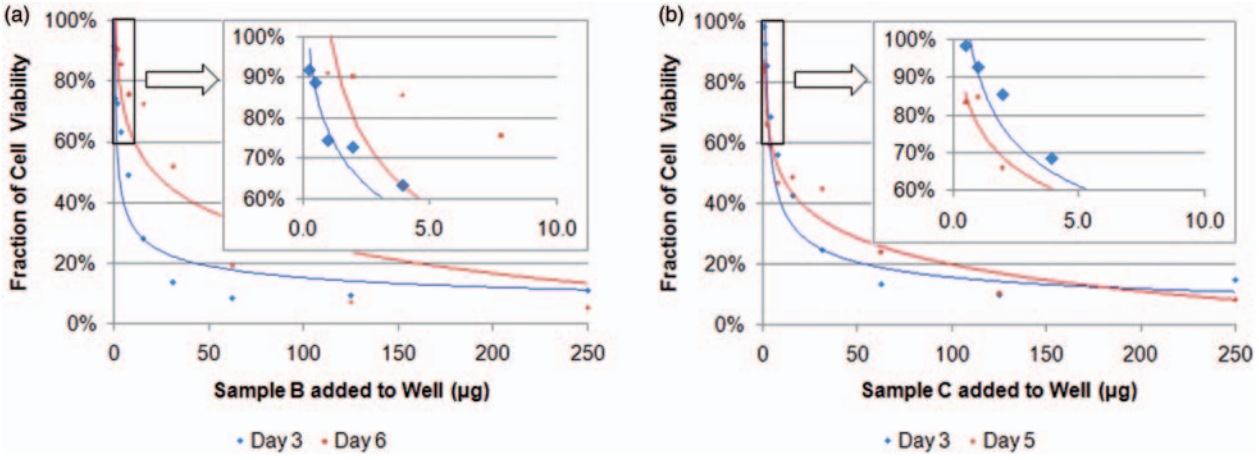

Figure 6. Inhibition of cell growth in medium containing (a) Sample B and (b) Sample C.

especially at higher concentrations and longer period of interaction. To distinguish MNP and PLGA for cytotoxicity, the drug delivery of MNC containing PLGA matrix but without MNP was tested for its biocompatibility (Figure 6(b)). The cell inhibition curves are almost identical. This suggests that $(Co_{0.5}Zn_{0.5})Fe_2O_4$ nanoparticles contribute very little to the cytotoxicity. PLGA is a widely used biodegradable polymer for drug delivery carrier because of its biocompatibility, long-standing track record in biomedical applications, and well-documented utility for sustained drug release.[8,14] Indeed, PLGA is approved by Food and Drug Administration for clinical use, but further investigation is warranted for the fabrication of the drug delivery spheres to promote its biocompatibility.

Conclusions

The fraction cell viability has been characterized by MTT assay for four different specimens at different additions by weight. Sample A consists of 10% $(Co_{0.5}Zn_{0.5})Fe_2O_4$, 20% 5-FU, and 70% PLGA. Sample B consists of 10% $(Co_{0.5}Zn_{0.5})Fe_2O_4$ and 90% PLGA, and Sample C consists of PLGA alone. Also, a positive cell viability by MTT assay was done for 5-FU. The specimens were tested to evaluate the drug release pattern of the system and the biocompatibility of the MNC components. As is expected, the major cytotoxicity component of the MNC is the chemotherapy agent, and it appears that drug-containing MNC possesses more potent cell inhibition effect than the chemodrug alone group at lower concentrations (<1 µg). It is postulated that the increased therapeutic efficacy may be due to the efficiency of MNC uptake and the sustained drug release. While the fraction of cell viability of the cells with MNC containing PLGA only and the PLGA plus cobalt zinc ferrite nanoparticles is reasonably comparable; further exploration is underway to address this issue.

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