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Polyhydroxybutyrate-Coated Magnetic Nanoparticles for Doxorubicin Delivery: Cytotoxic Effect Against Doxorubicin-Resistant Breast Cancer Cell Line

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In this study, polyhydroxybutyrate (PHB)-coated magnetic nanoparticles (MNPs) were prepared by coprecipitation of iron salts (Fe^{2+} and Fe^{3+}) by ammonium hydroxide. Characterizations of PHB-coated MNPs were performed by Fourier transform infrared spectroscopy, x-ray diffraction, dynamic light scattering, thermal gravimetric analysis, vibrating sample magnetometry, and transmission electron microscopy analyses. Doxorubicin was loaded onto PHB-MNPs, and the release efficiencies at different pHs were studied under in vitro conditions. The most efficient drug loading concentration was found about 87% at room temperature in phosphate-buffered saline (pH 7.2). The drug-loaded MNPs were stable up to 2 months in neutral pH for mimicking physiological conditions. The drug release studies were performed with acetate buffer (pH 4.5) that mimics endosomal pH. Doxorubicin (60%) released from PHB-MNPs within 65 hours. Doxorubicin-loaded PHB-MNPs were about 2.5-fold more cytotoxic as compared with free drug on resistant Michigan Cancer Foundation-7 (human breast adenocarcinoma, MCF-7) cell line (1 μM doxorubicin) in vitro. Therefore, doxorubicin-loaded PHB-MNPs lead to overcome the drug resistance.

Keywords: PHB, doxorubicin, magnetic nanoparticles, targeted drug delivery, drug resistance

INTRODUCTION

Delivering drugs into targeted tissues by engineered nanoparticles (NPs) is one of the recent concerns in medicine. The most important characteristics for a successful nanoparticle-mediated drug delivery are stability, higher loading capacity of nanoparticles, and the flexibility of carrying various therapeutics.[1] Nanoparticle-mediated drug delivery is advantageous in delivering drugs that are not easily soluble and readily metabolized when administered by other methods, in conveying drugs into targeted tissues and in reduction of toxicity while maintaining therapeutic effects of delivered drugs.[2,3]

Magnetic nanoparticles (MNPs), which commonly consist of magnetic elements such as iron, nickel, and cobalt, are widely studied for their applications in medicine such as enzyme and protein immobilization, magnetic resonance imaging, RNA and DNA purification, and magnetic cell separation.[4–8] The MNPs are one of the most efficient drug delivery systems for cancer therapy because of their ability of being manipulated under magnetic field. Especially, iron oxide nanoparticle–based therapy represents an important alternative to conventional chemotherapy, radiation, or surgery. Surrounding the iron core is generally a polymer coating that serves as a protective layer and also for the

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attachment of different moieties, such as drugs, antibodies, and vitamins, for targeting or additional imaging purposes.[9] Polymeric coating materials can be either synthetic or natural. Polymers based on poly(ethylene-co-vinyl acetate) (PEVA), poly(vinylpyrrolidone) (PVP), poly(lactic-co-glycolic acid) (PLGA), poly(ethyleneglycol) (PEG), poly(vinyl alcohol) (PVA), and others are typical examples of synthetic polymeric systems.[10–12] Natural polymer systems include use of gelatin, dextran, chitosan, pullulan, and others.[13–17] Although a number of synthetic biodegradable polymers have been developed for biomedical applications, the use of natural biodegradable polymers remains attractive because of their abundance in nature, biocompatibility, and ability to be readily modified.[18]

Polyhydroxyalkanoates (PHAs) are aliphatic polyesters produced by a wide range of microorganisms as intracellular carbon and energy storage compounds.[19] PHAs have received significant interests because of its biocompatible and biodegradable properties with potential applications in drug delivery and biomedical applications.[20] A stable and efficient immobilization of a specific ligand to the surface of PHA nanoparticles is necessary for the production of targeted drug carrier system.

Among PHAs, particular attention has been focused on the use of polyhydroxybutyrate (PHB) and related copolymers.[21] Hydrolytic degradation of PHB in vitro proceeds to the monomer D-3-hydroxybutyricacid that is a normal constituent of blood and constitutes one of the 3 ketone bodies produced endogenously by the ketogenesis process. It is, therefore, thought that PHB can be well tolerated in vivo.[22]

Doxorubicin is an anthracycline antibiotic. It is a commonly used anticancer agent in many cancer types, and its most serious side effect is damaging the heart cells.[23] Therefore, targeting of this chemotherapeutic agent with a suitable nanocarrier-mediated drug delivery system is important.

In this study, novel synthesized PHB-coated MNPs (PHB-MNPs), which have targeting potential under magnetic field, were characterized by FTIR, DLS, XRD, TGA, VSM, and TEM analyses. Doxorubicin was loaded onto the PHB-MNPs. Loading efficiencies and drug releases were investigated at different pHs. Also, in vitro cytotoxicity analysis of doxorubicin-loaded PHB-MNPs was performed on doxorubicin-resistant Michigan Cancer Foundation-7 (human breast adenocarcinoma) MCF-7 cell line.

MATERIALS AND METHODS

Iron (II) chloride tetrahydride ($FeCl_2.4H_2O$) and iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) were obtained from Merck (Germany); polyhdroxybutyrate (PHB) and ammonium hydroxide (NH_4OH), RPMI-1640 medium, fetal bovine serum, trypsin–EDTA, phosphate-buffered saline (PBS), gentamicin, Tris, and acetic acid were purchased from Sigma–Aldrich (Chemie GmbH, Germany). XTT cell proliferation assay kit was supplied by Biological Industries (Israel Beit Haemek LTD). Doxorubicin was kindly provided from Gülhane Military Academy, School of Medicine (Ankara, Turkey). MCF-7 cell line was obtained from SAP Institude (Ankara, Turkey), and 1-μM doxorubicin-resistant MCF-7 cell line was developed by Kars et al.[23]

In situ synthesis of PHB-coated magnetic iron oxide nanoparticles

PHB-coated magnetic iron oxide nanoparticles (MNP) were in situ synthesized by the coprecipitation of Fe (II) and Fe (III) salts in the presence of PHB molecules with some modifications of Xiong et al.[24]

Iron salts (1.34 g of $FeCl_2$ $4H_2O$ and 3.40 g of $FeCl_3$ $6H_2O$) were dissolved in 30 mL of 1% PHB solution under the nitrogen gas flow and by vigorously stirring at 2500 rpm. The ammonia solution (32%, NH_4OH) was added very slowly to produce smaller sized nanoparticles. The resulting solution was stirred for an additional 2–3 hours. The colloidal PHB-MNPs were extensively washed with ethanol and separated by magnetic decantation for several times.

Characterization of PHB-coated magnetic iron oxide nanoparticles

Crystal structures of synthesized PHB-MNPs were analyzed by x-ray diffraction (XRD). The chemical groups and chemical interactions involved in synthesized PHB-MNPs were identified using the Fourier transform infrared spectroscopy (FTIR) methods. The sizes of magnetic core and morphological properties were observed through transmission electron microscopy (TEM) images. The hydrodynamic sizes were determined with dynamic light scattering (DLS) measurements. The qualitative and quantitative information about the volatile compounds of the nanoparticles have been provided by thermal gravimetric analysis (TGA). Magnetic properties of PHB-MNPs were determined through vibrating sample magnetometry (VSM) analyses.

Doxorubicin loading on PHB-coated magnetic iron oxide nanoparticles

Loading studies of doxorubicin was carried out in PBS (pH 7.2), in different drug concentrations (200, 250, 400, 500, and 600 μg/mL). The mixture of buffer, drug, and PHB-MNPs was rotated for 24 hours in a light-protected tube. After the rotation, doxorubicin-loaded PHB-MNPs were separated by magnetic separation,

and the doxorubicin-loading efficiency was quantified by measuring the absorbance values at 481 nm by a UV-spectrophotometer (equation 1). The loading or binding of maximum doxorubicin concentration to the PHB-MNPs was confirmed by FTIR analysis.

$$\text{Loading Efficiency } (\%) = \frac{(\text{total } \mu\text{g of drug added}) - (\mu\text{g of drug in supernatant})}{(\text{total } \mu\text{g of drug added})} \times 100 \quad (1)$$

Stability of doxorubicin on PHB-coated magnetic iron oxide nanoparticles

Stabilities of drug-loaded PHB-MNPs were studied in PBS buffer (pH 7.2) at 37°C up to 8 weeks. Doxorubicin stabilities were measured as the absorbance values at 481 nm by a UV spectrophotometer.

Release of doxorubicin from PHB-coated magnetic iron oxide nanoparticles

The release of doxorubicin (400 and 500 μg/mL) from PHB-MNPs was analyzed in acetate buffer with pH 4.5 up to 65 hours. The amount of released doxorubicin was determined by the decrease in the absorbance of the solution at 481 nm by a UV spectrophotometer.

Cellular internalization of PHB-coated magnetic iron oxide nanoparticles

Doxorubicin-loaded PHB-MNPs (400 μg/mL) were incubated for 24 hours at 37°C with doxorubicin-resistant MCF-7 cell line in 6-well plates. Then, the cells were washed 3 or 4 times with PBS. Cells' photographs were taken by fluorescence microscopy after 4 hours to determine their cellular internalization.

Cytotoxicity of PHB-coated magnetic iron oxide nanoparticles

Doxorubicin-resistant MCF-7 cells were used in the cytotoxicity analysis. Cells were grown in 75T culture flasks in RPMI 1640 culture medium supplemented with 10% fetal bovine serum and 1% gentamycin solution at 37°C under 5% CO_2. The cells were subcultured 2–3 times per week with 0.25% trypsin–EDTA.

Antiproliferative effects of doxorubicin-loaded PHB-MNPs on MCF-7 cells were evaluated by means of the XTT Cell Proliferation Kit (Biological Industries, Israel) according to manufacturer's instructions. MCF-7 cells were seeded into 96-well microtiter plates (Greiner) at a concentration of 5.0×10^4 cells/well and incubated for 72 hours in the presence of different dilutions of nanoparticles. At the end of 72 hours, XTT reagent was added, and soluble product was measured at 500 nm with Spectromax 340 96-well plate reader (Molecular Devices).

RESULTS

The synthesis conditions of PHB-MNPs were optimized, and characterization of synthesized PHB-MNPs was performed by FTIR (Figure 1), TEM (Figure 2), TGA, and VSM analyses.

In Figure 1, FTIR results showed the presence of magnetite in doxorubicin-loaded PHB-coated nanoparticles (583 cm^{-1}). A strong peak at 1728 cm^{-1} indicates the stretching of C=O ester bond, which is the characteristic of PHB. The other peaks obtained at 1285 m^{-1} (corresponding to C= H stretch) and 1182 cm^{-1} (corresponding asymmetric C= O= C bridge) show characteristic peaks for PHB. The FTIR spectra of free doxorubicin and doxorubicin-loaded PHB-MNPs were also shown in Figure 1. The peaks at 1615 and 1576 cm^{-1} were indicated that doxorubicin was loaded to PHB-MNPs.

Size and morphology of synthesized PHB-MNPs were observed by TEM. Images showed that the synthesized PHB-MNPs were almost spherical and had more uniform size distribution (Figure 2). Figure 3 shows a schematic representation of doxorubicin-loading onto PHB-MNPs.

The amount of PHB in nanoparticles was measured by thermo-gravimetric analyzer. The PHB-MNPs gave their distinctive TGA curves, which can provide indications of the content of PHB polymer. From the result, it was calculated that the percentage of PHB was around 80% (wt/wt) and the amount of magnetite was around 20% (wt/wt).

Magnetic hysteresis curve was obtained by vibrating sample magnetometer. The applied magnetic field was changed, and magnetization properties of synthesized Fe_3O_4 and PHB-coated Fe_3O_4 nanoparticles were measured at 37°C. Remanence and coercivity were not observed in the hysteresis curve. This phenomenon proved that all nanoparticles synthesized in this study are superparamagnetic, which show magnetic properties only in the presence magnetic field.[25]

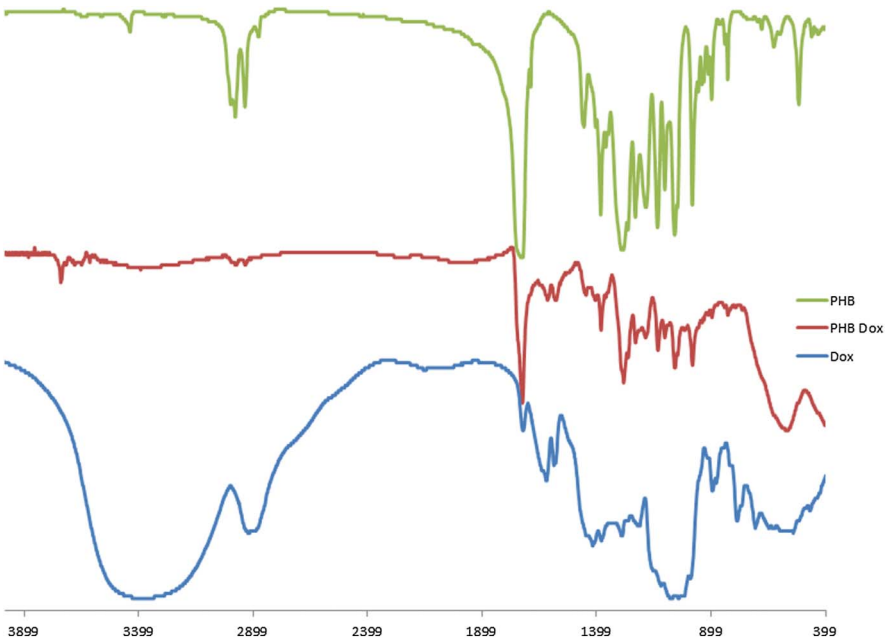

FIGURE 1. FTIR analysis of PHB, doxorubicin-loaded PHB-MNPs, and free doxorubicin.

Doxorubicin loading studies were performed onto PHB-MNPs in PBS buffer (pH 7.2). To find the most efficient drug loading capacity on PHB-MNPs, doxorubicin concentration gradually increased up to 600 μg/mL where the loading efficiency started to decrease. The most efficient drug loading concentration was found as 400 μg/mL. The 400-μg/mL doxorubicin-loading efficiency of PHB-MNPs was 348 μg/mL (%87) in PBS buffer at pH 7.2 (Figure 4).

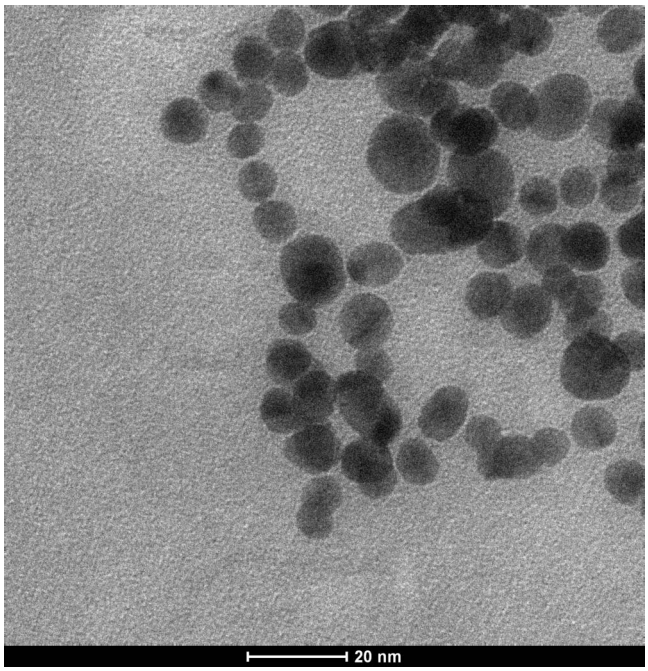

FIGURE 2. TEM analyses of PHB-MNPs.

The stability of doxorubicin-loaded PHB-MNPs was studied in PBS buffer (pH 7.2) at 37°C that mimics physiological conditions. The results showed that about 35% of the doxorubicin released within 2 weeks. However, 65% of the drug was not released at pH 7.2 up to 8 weeks.

The most efficiently loaded drug concentrations (400 and 500 μg/mL) were selected, and release studies were performed in acetate buffer at pH 4.5 that mimics endosomal conditions. The release studies were continued up to 65 hours. The release rate of 500-μg/mL doxorubicin-loaded PHB-MNPs was faster as compared with 400-μg/mL doxorubicin. About 30% of drug released from 500-μg/mL doxorubicin-loaded PHB-MNPs in the first hour, and 50% of the drug released up to 9 hours. However, only 10% of doxorubicin released from 400-μg/mL doxorubicin-loaded PHB-MNPs, and 50% of the drug release was achieved after 25 hours. About 60% of doxorubicin released from both in 65 hours (Figure 5).

The cellular internalization of doxorubicin-loaded PHB-MNPs was demonstrated by fluorescence microscopy (Figure 6). The results confirmed that nanoparticles could be internalized into doxorubicin-resistant MCF-7 cells.

The cytotoxic effect of doxorubicin-loaded PHB-MNPs on resistant MCF-7 cells was investigated by XTT cell proliferation assay, and IC_{50} values were calculated. In this study, empty PHB-MNPs were found

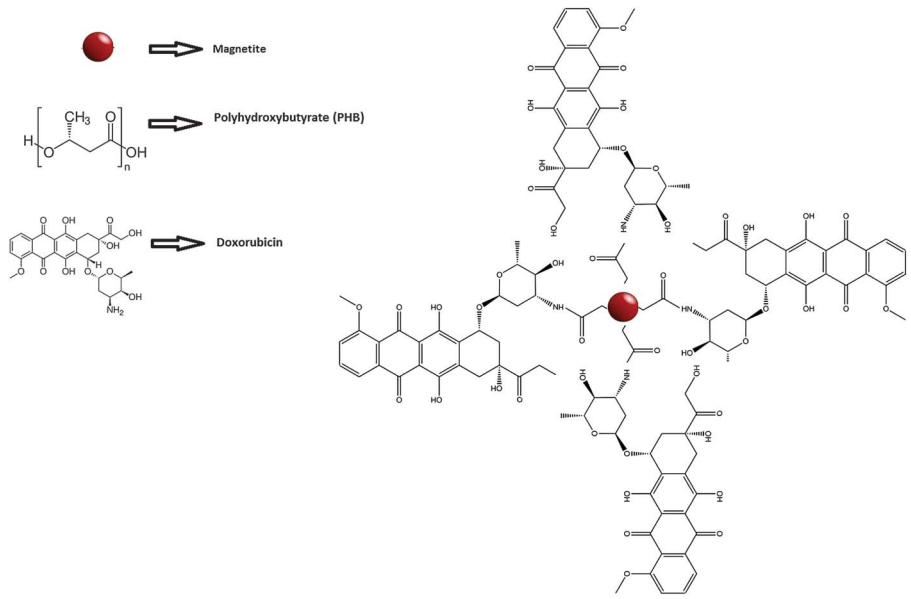

FIGURE 3. Schematic representation of doxorubicin loading onto PHB-MNPs.

not significantly cytotoxic up to 500 μg/mL (Figure 7). The IC_{50} value of minimum and maximum doses of doxorubicin (200 and 400 μg/mL) loaded PHB-MNPs were found as 89 and 70 μM on doxorubicin-resistant MCF-7 cells, respectively (Figure 8 and Figure 9). Previously, Kars et al[23] reported the IC_{50} value as 183 μM for 1-μM doxorubicin-resistant MCF-7 cells by applying free doxorubicin. From the IC_{50} values, it was seen that the loading of doxorubicin onto PHB-MNPs increased its efficiency nearly up to 2- to 2.5-fold.

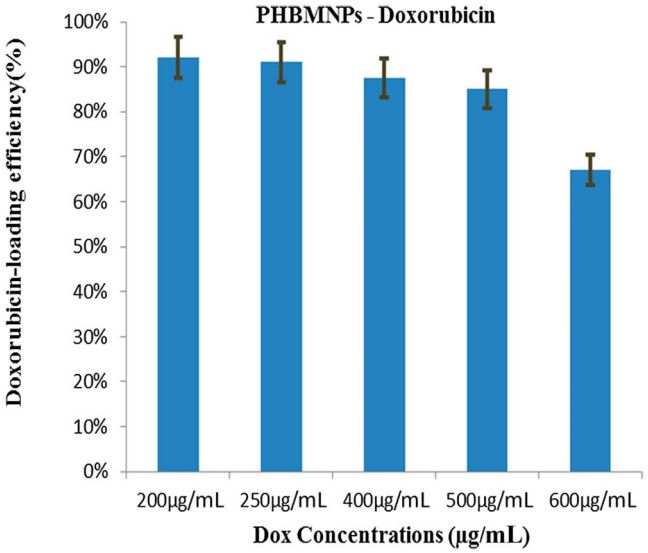

FIGURE 4. Loading efficiencies of different concentrations of doxorubicin to PHB-MNPs in pH 7.2 PBS buffer.

DISCUSSION

MNPs can be used efficiently in targeting of anticancer drugs to the tumor site by using external magnetic field.[26,27] Biopolymers such as polyhydroxyalkanoates (PHAs) are favorable alternative for the nanoparticular systems due to their biodegradable nature and biocompatibility.[19] Poly(3-hydroxybutyrate), as a member of PHA family, has been studied extensively in drug delivery applications.[27] In this study, PHB-coated iron oxide nanoparticles (PHB-MNPs) were synthesized and loaded with doxorubicin. The magnetic PHB nanoparticles were characterized, and most efficient doxorubicin loading and release conditions have been optimized. In addition, in vitro cytotoxicity of these nanoparticles was evaluated with doxorubicin-resistant MCF-7 cell line.

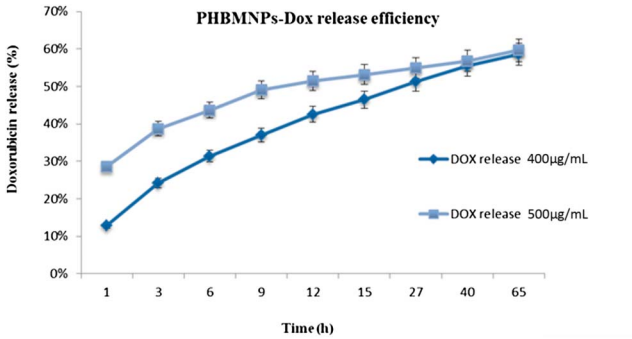

FIGURE 5. Release of doxorubicin from PHB-MNPs at pH 4.5.

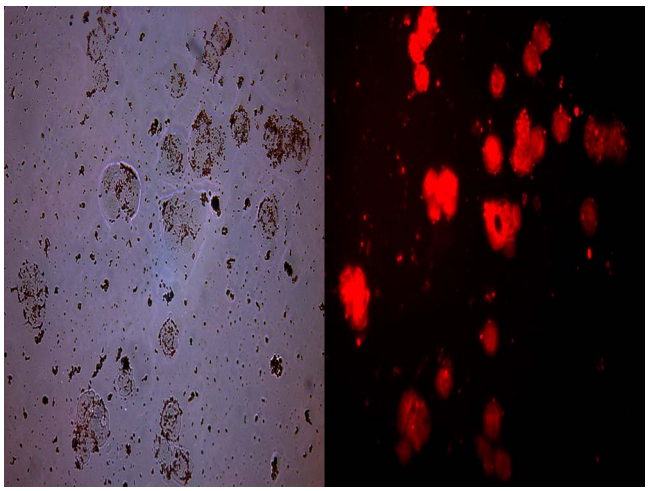

FIGURE 6. Cellular internalization of 400-μg/mL doxorubicin-loaded PHB-MNPs by fluorescence microscopy (×20).

Initially, synthesized PHB-MNPs were characterized by FTIR, TGA, VSM, and TEM. The average diameters of PHB-MNPs were around and 30–35 nm. In the literature, PHB-coated MNPs were previously synthesized in the range of 240–400 nm.[28] For biomedical applications, the size of the nanoparticles is critical because particles on the scale of 10–20 nm are efficiently captured and eliminated for the clearance by liver.[29] The size of the nanoparticles being in the range 10–35 nm makes their circulation into blood vessels feasible.[30] The size of the synthesized PHB-MNPs in this study was optimum for biomedical applications. The results of TGA showed that the percentage of PHB around the magnetite core was 80%. This result shows that PHB coating efficiency was higher with respect to literature. Previously, Unsoy et al[31] showed that chitosan coating efficiency as 23% on iron oxide nanoparticles. A strong peak at 1728 cm^{-1} indicates the presence of PHB. Previously, Hong et al[32] reported the use of FTIR in determining the presence of PHB polymer in the environment. PHB-MNPs were loaded with model drug, doxorubicin. In the FTIR studies, the characteristic peaks obtained from doxorubicin were compared with the peaks resulted from doxorubicin-loaded PHB-MNPs. Peaks at 1615 and 1576 cm^{-1} were indicated that doxorubicin was successfully loaded onto the nanoparticles.

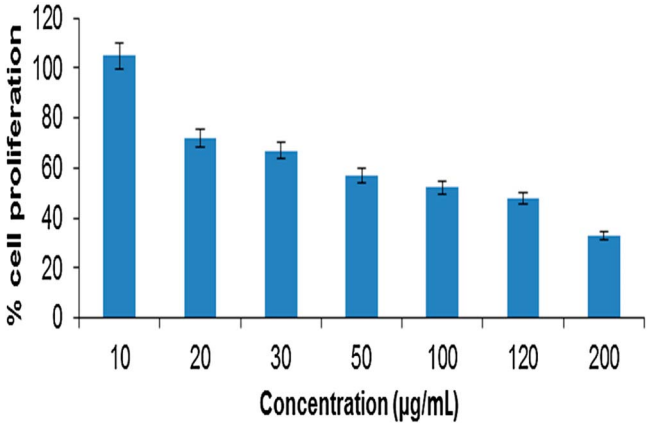

FIGURE 8. Cytotoxic effect of 200-μg/mL doxorubicin-loaded PHB-MNPs on 1-μM doxorubicin-resistant MCF-7 cells.

Doxorubicin loading studies were performed in PBS buffer (pH 7.2). Doxorubicin and PBS have similar molecular structures.[33] Therefore, the drug can be easily dissolved and loaded onto PHB-MNPs in PBS at neutral pH, which mimics physiological conditions. To find out the saturated loading capacity, doxorubicin concentration gradually increased up to 600 μg/mL. However, the most efficient drug loading concentration was

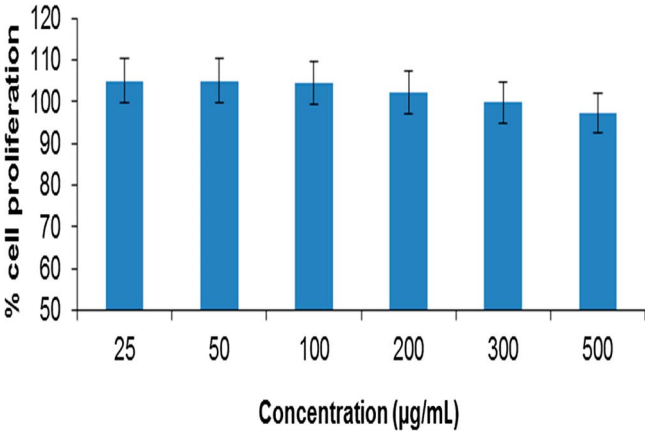

FIGURE 7. Cytotoxic effect of PHB-MNPs on 1-μM doxorubicin-resistant MCF-7 cells.

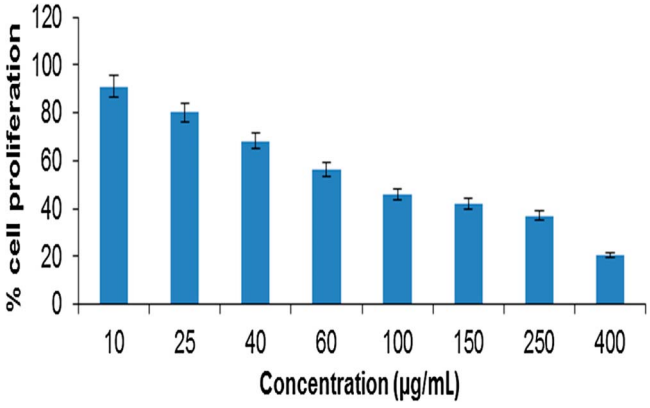

FIGURE 9. Cytotoxic effect of 400-μg/mL doxorubicin-loaded PHB-MNPs on 1-μM doxorubicin-resistant MCF-7 cells.

obtained as 400 μg/mL with 87% efficiency. Previously, 400-μg/mL doxorubicin was loaded onto PAMAM dendrimer–loaded MNPs with 96% efficiency.[34] Doxorubicin loading efficiency onto PHB-MNPs was efficient and similar to the literature. The maximum drug loading amount was directly related to the shape and size of internal cavities of the polymer. Loading efficiency started to decrease after 500-μg/mL doxorubicin where all the cavities of the polymer started to be filled out with the drug.

Doxorubicin-loaded PHB-MNPs were studied up to 8 weeks in PBS buffer and found stable. This is a desirable property, which provides an advantage in the storage. As it was also emphasized in the literature, the stability of drug loaded MNPs is a very important parameter for the preservation of the drug in the active form throughout its shelf-life.[35] The instability of drug-loaded MNPs would lead to the release of drug before reaching to the target site.[36]

The release efficiencies of doxorubicin-loaded PHB-MNPs were investigated at acetate buffer (pH 4.5) that mimics endosomal conditions. The release of 500-μg/mL doxorubicin was faster as compared with 400-μg/mL doxorubicin within 40 hours. 60% of doxorubicin released from both in 65 hours. The surface area of the nanoparticles, the size and intensity of the pores, irregularities of particles that may occur during the production process, factors causing degradation of the polymer, the drug content, and the size of the particles are the important factors that may affect drug release from the particles.[37,38] In this study, initial drug release in the first hour can be explained by the burst effect. However, it is only 10% for the 400-μg/mL doxorubicin-loaded PHB-MNPs. The release rate was slower after 9 hours. The slow release of the drug at approximately late endosomal and lysosomal pH (4–4.5) makes PHB-MNPs more attractive for doxorubicin delivery since doxorubicin could be successfully delivered to the late endosome near to nucleus of the cells where it shows anticancer activity. Previously, Erdal et al loaded etoposide onto Fe_3O_4-loaded PHB nanoparticles and investigated in vitro release of the drug. The release rate of etoposide was significantly higher in the first few hours. This burst release effect was found to be normal as the most of the etoposide was onto the surface of the nanoparticle.[28] Also, Gaucher et al[39] had observed that at the initial time point, 30%–35% of the etoposide was significantly reduced with burst effect from poly(N-vinylpyrrolidone)-block-poly(D, L-lactide) nanoparticles.

Cell–nanoparticle interaction and in vitro cytotoxicity of doxorubicin-loaded PHB-MNPs were evaluated by using 1-μM doxorubicin-resistant MCF-7 cell line. The cellular internalization of doxorubicin-loaded PHB-MNPs was visualized by fluorescence microscopy. Biodegradation and tissue response to PHB have been evaluated by Gogolewski et al.[40] They observed no necrosis, inflammation, or abscess formation with subcutaneous implantation of PHB. Their study suggested that PHB is well tolerated in vivo, and most of the polymer is degraded within 6 months. The tissue response to PHB microspheres still remains to be studied.

To quantify the cytotoxic effects of empty PHB-MNPs and doxorubicin-loaded PHB-MNPs on doxorubicin-resistant MCF-7 cells, XTT cell proliferation assay was performed. Free doxorubicin is a substrate of P-glycoprotein (multidrug resistance protein-1), which is pumped out of the cell membrane causing drug resistance in cancer cells.[41] By releasing the drug in low pH away from the cell membrane and efflux pumps, it will be possible to overcome the MDR-1–related resistance. Previously, Kars et al[23] reported the IC_{50} value of free doxorubicin for 1-μM doxorubicin-resistant MCF-7 cells as 183 μM. In this study, empty PHB-MNPs were not found significantly cytotoxic up to 500 μg/mL. About 200- and 400-μg/mL doxorubicin-loaded PHB-MNPs were found as nearly 2- and 2.5-fold more toxic compared with free doxorubicin, respectively. These results showed that doxorubicin-loaded PHB-MNPs are more effective over doxorubicin-resistant MCF-7 cell line and cause to overcome the drug resistance.

In addition, it is known that free doxorubicin represents a dose-dependent and cumulative cardiotoxicity as one of the most serious side effects.[42] Up to now, different therapies have been proposed to overcome or decrease the cardiotoxicity such as usage of metabolic/antioxidant agents and calcium antagonists.[43–45] However, these approaches may not completely prevent cardiotoxicity. In this study, loading of doxorubicin onto PHB-MNPs increases the efficacy by targeted delivery of drug to the tumor site. Besides, magnetic targeting may decrease the overall toxicity of the drug by avoiding nonspecific delivery to the healthy cells. The decreased required dose of doxorubicin and dosing frequency results in a reduction at toxicity. Because of the alteration of dose scheduling of doxorubicin, the cardiotoxicity reduces as a means of reducing doxorubicin.[46] The reduced toxicity could be due to the gradual release of doxorubicin from the PHB-coated nanoparticles or as a result of reduced exposure as the drug-loaded nanoparticles are sequestrated at the tumor site.[47] However, this study has still some limitations because these are the results of an in vitro study. Cardiotoxicity can be increased clinically due to an increased myocardial cell uptake as a result of myocyte injury. Further

clinical studies may be necessary to display the cardiotoxic effect of doxorubicin-loaded PHB-coated MNPs.

CONCLUSIONS

MNPs, which were coated with PHB, can be used to target specific cancer cells in the presence of magnetic field. Size, PHB coating ratio, and doxorubicin release efficiency at various pHs make PHB-MNPs attractive as a targeted drug delivery system. Doxorubicin was used as a model anticancer agent and loaded onto PHB-MNPs. Doxorubicin, being a DNA-targeting drug, needs to be released near the nucleus. PHB-MNPs loaded with doxorubicin releases most of the drug in low pH and is stable in physiological conditions. In addition, application of doxorubicin-loaded PHB-MNPs may help to overcome drug resistance in doxorubicin-resistant MCF-7 cells. Controlled release of doxorubicin from PHB-MNPs seems to be a promising candidate for targeted cancer therapy, overcoming drug resistance, and cardiotoxicity.

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