THE CYTOTOXICITY ANALYSIS OF PHB COATED MAGNETIC NANOPARTICLES ON SENSITIVE AND DOXORUBICIN RESISTANT MCF-7 CELL LINES

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Abstract
Biodegradable polymeric magnetic nanomaterials gained importance in biomedical and bioengineering research such as drug targeting, tissue engineering, cancer diagnosis and therapy. Polyhydroxybutyrate (PHB) is a nontoxic, biodegradable, biocompatible polymer and hence is suitable for medical applications. In this study, the cytotoxic effect of PHB coated magnetic nanoparticles were compared on sensitive and doxorubicin resistant MCF-7 cells. Resistant MCF-7 cell line was generated by applying doxorubicin in dose increments to sensitive cells. The synthesized nanoparticles were tested for their cytotoxic effects onto both sensitive and resistant cells. Then, PHB coated nanoparticles were applied onto cell lines and their uptake by the cells were observed with light microscopy. Cytotoxicity of PHB coated magnetic nanoparticles was investigated by XTT cell proliferation assay. The results demonstrated that synthesized nanoparticles were successfully uptaken by the cells and they were non-toxic to both sensitive and Doxorubicin resistant MCF-7 cells.

Keywords: PHB coated magnetic nanoparticles, Cytotoxicity, MCF-7 cell line.

1. INTRODUCTION
Nanoparticles used for anticancer drug delivery can be made from a variety of materials, including polymers, dendrimers, liposomes, and metals such as iron oxide. Polyhydroxyalkanoates (PHAs) are linear biodegradable polymers synthesized by bacteria. PHAs can be produced by many different bacterial strains [1]. They are naturally synthesized by bacteria as a storage molecule in response to conditions of physiological stress. There are many types of PHAs such as PHB (Poly-3-hydroxybutyrate), PHV (Poly3-hydroxyvalerate), PHHx (Poly 3-hydroxyhexanoate). Among them PHB is the most widely used member of this group [2]. There are many properties of PHB that enable us to use them in drug delivery systems. First, PHB is biodegradable and it is known that they can be degraded by PHA hydrolases and PHA depolymerases [1, 3]. Second, PHB are biocompatible and no toxic effect is observed during in vivo applications [4]. In this study, PHB coated magnetic nanoparticles were prepared and cytotoxicity of PHB coated magnetic nanoparticles was investigated by XTT cell proliferation assay on sensitive and Doxorubicin resistant MCF-7 cells.

2. MATERIAL AND METHODS

2.1 Materials
Iron (II) chloride tetrahydrhide (FeCl\textsubscript{2}.4H\textsubscript{2}O), iron (III) chloride hexahydrhide (FeCl\textsubscript{3}.6H\textsubscript{2}O) were obtained from Merck Germany; Polyhydroxybutyrate (PHB) and ammonium hydroxide (NH\textsubscript{4}OH), FITC (Fluorescein isothiocyanate), RPMI-1640, FBS, Trypsin–EDTA PBS(Phosphate buffered saline), Gentamicin, were purchased from Sigma-Aldrich Chemie GmbH, Germany. XTT cell proliferation assay kit (XTT) was supplied by Biological Industries, Israel Beit Haemek LTD.
2.2 Synthesis of PHB coated Magnetic Iron Oxide Nanoparticles

PHB coated magnetic iron oxide nanoparticles were in situ synthesized by the co-precipitation of Fe (II) and Fe (III) salts in the presence of PHB molecules with some modifications of Xiong et al [5]. Iron salts (FeCl₂ 4H₂O and FeCl₃ 6H₂O) were dissolved in PHB solution. Under the nitrogen (N₂) gas flow and by vigorously stirring at 3000 rpm. The ammonia solution (NH₄OH) was added very slowly to produce smaller sized nanoparticles. The colloidal PHB coated magnetic Fe₃O₄ nanoparticles were extensively washed with ethanol and separated by magnetic decantation for several times.

2.3 Characterization of Bare and PHB Coated Magnetic Nanoparticles

Crystal structures of synthesized MNPs were analysed by XRD. The chemical groups and chemical interactions involved in synthesized MNPs were identified using the FTIR methods.

2.4 Cellular internalization of PHB coated nanoparticles

PHB coated iron oxide nanoparticles were incubated with breast cancer (sensitive and resistance MCF-7) cell lines in 6 well plates and their photographs (scattering light microscope) were taken with time intervals during the incubation to determine their cellular internalization. In addition, PHB-MNPs were conjugated with fluorescent FITC which were applied onto breast cancer (MCF-7) cell lines. The resultant FITC-conjugated magnetic nanoparticles were visualized by confocal microscopy.

2.5 Cytotoxicity of PHB coated nanoparticles

Sensitive (MCF-7) and resistant (MCF-7/1000nMDox) human breast cancer cells were used for the cell studies. Cells were grown in culture flasks in RPMI/1640 culture medium supplemented with FBS, and gentamycin solution under CO₂ gas. Antiproliferative effects of PHB coated nanoparticles on MCF-7 cells were evaluated by means of the Cell Proliferation Kit (Biological Industries) according to manufacturer’s instructions. Assay was a colorimetric test based on the reduction tetrazolium salt, XTT to colored formazan products by mitochondria of live cells. In each plate assay was performed with a column of blank medium control and a cell control coloumn. Then, XTT reagent was added and soluble product was measured at 500 nm with Spectromax 340 96-well plate reader (Molecular Devices, USA).

3. RESULTS

3.1 Characterization PHB coated nanoparticles

The crystal structure of synthesized iron oxide (Fe₃O₄) nanoparticles was determined by XRD. Diffraction peaks observed are characteristic peaks of the magnetite (Fe₃O₄) crystal having an inverse cubic spinel structure by comparison with standards in Fig. 1.

![X-Ray powder Diffraction of synthesized iron oxide (Fe3O4) nanoparticles in different temperatures](image-url)
FTIR study showed the presence of poly-hydroxybutyrate in this structure of magnetic nanoparticle (Fig. 2).

**Fig. 2** FT-IR spectra of PHB coated iron oxide nanoparticle

### 3.2 Cellular internalization of PHB coated magnetic nanoparticles

In Fig. 3 and 4 it is demonstrated by light scattering microscopy that most of the PHB magnetic nanoparticles are taken up by the cells.

**Fig. 3** Cellular internalization of PHB coated magnetic nanoparticles by light scattering microscopy (X20) (A: 6.25 μg/ml, B: 12.5 μg/ml, C: 25 μg/ml, D: 50 μg/ml, E: 100 μg/ml, F: Control).
3.3 XTT assay- PHB coated nanoparticles effect on cell proliferation

In Fig. 5 it is showed that the effect of PHB coated nanoparticles on cell proliferation of MCF/7.

![Fig. 5 The effect of PHB coated nanoparticles on cell proliferation of MCF/7.](image)

**Fig. 5** The effect of PHB coated nanoparticles on cell proliferation of MCF/7.

4. DISCUSSION

Magnetic nanoparticles are widely used in biomedical applications, yet questions remain regarding the effect of nanoparticle and coating on nanoparticle cytotoxicity. In present study, PHB coated magnetic nanoparticles were synthesized and characterized. Cytotoxicity of PHB coated magnetic nanoparticles was investigated by XTT cell proliferation assay on sensitive and Doxorubicin resistant MCF-7 cells. XRD results revealed the presence of the Fe$_3$O$_4$ crystals in the synthesized nanoparticles bare or PHB coated. Even the peak positions were unchanged, which illustrated that the PHB binding process did not result in the phase change of Fe$_3$O$_4$. No evidence of impurities was found in the XRD pattern. The peaks shown in the XRD pattern of the prepared sample are sharp and intense, indicating crystallinity of the sample. In order to confirm the chemical composition of synthesized nanoparticles, FTIR spectra were obtained. The peak located in the 583 cm$^{-1}$ region, characteristic for the Fe-O group, is found in bare and PHB coated nanoparticle’s spectra, confirming that the products contain magnetite. All characteristic peaks
of PHB and iron oxide were present in the spectrum of PHB-MNP [6]. FTIR results showed the presence of magnetite in nanoparticle.

Endocytosis is known as the main mechanism of cellular internalization for the magnetic nanoparticle vectors [7]. In Fig. 3 and 4 it is demonstrated by light scattering microscopy that most of the PHB magnetic nanoparticles are taken up by the cells. The results are promising due to the fact that, nanoparticles can be internalized into the cells even if they are applied at low concentrations. Cellular internalization was carried out with five different concentrations of PHB coated nanoparticles and a control group. Cell viability was not affected.

Cytotoxicity of PHB magnetic nanoparticles was investigated by XTT cell proliferation assay. Survival rates indicated that there is no cytotoxic effect of the nanoparticles. Cells grown in same medium without any nanoparticle addition was the control group. Their proliferation was taken as 100% (Fig. 5). The results of toxicity assay showed that introduction of PHB coated iron oxide nanoparticles did not affect the cell growth. Cells showed excellent growth even in the highest dose of nanoparticles. In the literature, it has been shown that PHB has no cytotoxicity because it is a natural biopolymer and it can be degraded by enzymes in the body. In addition, growth stimulation of PHB nanoparticles was observed both in our study and in the literature [8].

5. CONCLUSION

In summary, the cell proliferation measurements showed that the PHB coated iron oxide nanoparticles did not have cytotoxicity on MCF-7 cells.

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LITERATURE