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Preparation and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHX) based nanoparticles for targeted cancer therapy

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ABSTRACT

Targeted drug delivery systems are one of the most promising alternatives for the cancer therapy. Rapid developments on nanomedicine facilitated the creation of novel nanotherapeutics by using different nanomaterials. Especially polymer based nanoparticles are convenient for this purpose. In this study; a natural polymer (poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), PHBHHX) was used as a base matrix for the production of a novel nanotherapeutic including antineoplastic agent, Etoposide and attached folic acid as a ligand on the nanoparticles. Modified solvent evaporation technique was used for the production of PHBHHX nanoparticles and the average size of the obtained PHBHHX nanoparticles were observed in the range of 180 nm and 1.5 μm by the change in experimental conditions (i.e., homogenization rate, surfactant concentration and polymer/solvent ratio). By the increase in homogenization rate and surfactant concentration, size of the nanoparticles was decreased, while the size was increased by the increase in polymer/solvent ratio. Drug loading ratio was also found to be highly affected by polymer/drug ratio. Surface charge of the prepared nanoparticles was also investigated by zeta potential measurements. In the cytotoxicity tests; Etoposide loaded and folic acid attached PHBHHX nanoparticles were observed as more effective on HeLa cells than Etoposide loaded PHBHHX nanoparticles without attached folic acid. The cytotoxicity of folic acid conjugated PHBHHX nanoparticles to cancer cells was found to be much higher than that of normal fibroblast cells, demonstrating that the folate conjugated nanoparticles has the ability to selectively target to cancer cells. In addition, apoptotic/necrotic activities were evaluated for all formulations of the PHBHHX nanoparticles and parallel results with cytotoxicity tests were obtained. These studies demonstrate that the folic acid attached and Etoposide loaded PHBHHX nanoparticles seem as promising for the targeted cancer therapy.

1. Introduction

Cancer is still one of the most destructive disease group for human body due to the complexity and progressive nature of the cancer diseases (Stewart and Kleihues, 2003). In the last decade, there are 10% of decrease in the death caused by cancer diseases by using conventional techniques (Eschenbach von, 2004). On the other hand conventional cancer treatment strategies such as surgery, chemotherapy, radiotherapy or immunotherapy which are widely used in all over the world do not seem satisfactory and do not supply selective therapy. Meaning that almost all of the mentioned strategies have very destructive side effects for the heathy cells and tissues which surround the cancerous tissues (Catane et al., 2006; Llombart et al., 2006; Gabriel, 2007). It is also desirable to maintain a steady infusion of the drug into the tumor interstitium to accomplish continuous extermination of the dividing cells that eventually results in tumor regression. Therefore cancer diseases still need novel and both effective diagnosis and treatment strategies. Development of suitable active agent delivery systems that carry the therapeutically active agent molecules only to the tumor site without affecting healthy organs and tissues has to be developed. At this point, nanotechnology plays an important role in therapies of the future as "nanomedicines" by enabling this situation to happen, thus lowering doses required for efficacy as well as increasing the therapeutic indices and safety profiles of new therapeutics (Koo et al., 2005).

Many different types of nanomaterials including liposomes, micelles, nanoemulsions, nanoparticulate systems (polymer, lipid, ceramic and albumin based nanoparticles and nanogels), dendrimers, carbon nanotubes and peptide–protein nanotubes are still under investigation for convenient drug delivery (Brigger et al.,

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2002; Hughes, 2005). Polymeric nanoparticles are one of the most popular group throughly the mentioned nanomaterials due to their easy production and process diversity into the required characteristics for the design of suitable drug delivery systems. Especially biocompatible and biodegradable polymeric structures such as natural polymers are preferred for this purpose.

Targeted delivery is a viable route to enhance intracellular uptake of drug containing nano-carriers within cancerous cells at the tumor site thereby increasing the effective use of the drug and minimizing undesirable side effects and toxicity. Various targeting moieties or ligands against tumor-cell specific receptors have been immobilized on the surface of polymeric nanoparticles to achieve active targeting. Among them, vitamin folic acid (folate) is a stable, inexpensive and nonimmunogenic chemical with a high affinity for the folate receptor which overexpresses on many human epithelial cancer cell surfaces such as uterus, colon, lung and ovary cancer (Mathew et al., 2010). Therefore conjugation of macromolecules or drugs can enhance drug uptake and targeting (Yang et al., 2010a,b).

Polyhydroxyalkanoates are bacteria-based natural polymers and they can be used in many different application fields due to their renewable nature and favorable biological characteristics (i.e., biocompatibility, biodegradability, easy processability into desired shape or geometry, cost effectiveness, etc.) which are required in these mentioned applications (Anderson and Dawes, 1990; Byrom, 1992; Zinn et al., 2001; Lee, 1996). In the last decade these polymers have been used in the production of certain drug delivery systems (Bayram et al., 2008). Among them poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) is a promising drug carrier, especially for hydrophobic drugs due to its ester backbone and alkane side chain. In addition to its total biodegradability and nontoxic-nonimmunogenic degradation products (i.e., 3-hydroxybutyrate), this polymer has better elastic properties (Lu et al., 2010a,b).

Etoposide (is also called vepesid, code designation VP-16-213, abbreviated VP16), a topoisomerase II inhibitor, is an important chemotherapeutic agent currently in clinical use and has a significant activity on different tumors such as lung, stomach, ovarian, testicular cancers and lymphomas (Hande, 1998). Its activity is mediated by its interaction with topoisomerase II, a nuclear enzyme which passes an intact helix through a transient double-stranded break in DNA to modulate DNA topology by using ATP. Following the strand passage, the DNA backbone is religated and the structure of DNA restored. Etoposide damage DNA by interaction with topoisomerase II to form cleavable complexes that prevent religation of DNA leading to double-strand DNA breaks in the genome of treated cells (Hande, 2006). Etoposide is poorly soluble in water and has a short biological half-life (3.6 h). As the effective chemotherapy depends on prolonged exposure of cancerous cells to anticancer agents, drug delivery systems has been used to deliver Etoposide with higher efficiency and also with fewer adverse side effects (Zhang et al., 2011; Dhanarajua et al., 2010; Reddy and Murthy, 2005).

In this study; PHBHHX was used for the production of anticancer agent loaded nanoparticles for targeted cancer therapy and the modified solvent evaporation technique was used for this purpose. In the active agent loading and release experiments Etoposide was used as a model anticancer agent and *in vitro* release studies were performed spectrophotometrically. Additionally folic acid was attached onto the PHBHHX nanoparticles as a ligand for cancer cell targeting. The physicochemical properties of nanoparticles were studied by FTIR, zetasizer, zeta potential, scanning electron microscopy (SEM) and atomic force microscopy (AFM). In the PHBHHX nanoparticle–cell interaction studies both cell cytotoxicity over the model healthy cells, mouse fibroblast-like cell line (i.e., L-929 cell line) and model cancer cells, human epithelial carcinoma cell line (i.e., HeLa cells) were investigated. Apoptosis and necrosis indexes were also evaluated.

2. Materials

PHBHHx [weight-average molecular weight (M_w) = 454,000] containing 12 mol% of 3-hydroxyhexanoate (3-HHx) units was supplied by Procter & Gamble Company (USA). Dichloromethane and Tween-80 were purchased from Merck (Germany). Tripsin-Ethylenediamine tetra acetic acid (Tripsin-EDTA), Dulbecco's Modified Eagle's Medium (DMEM F-12), Fetal Calf Serum (FCS) and DMSO (Dimethylsulfoxide) were purchased from Biological Industries (Israel). 3-(4,5-Dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Serva (USA). Folic acid and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were purchased from Sigma (USA). Hoechst dye 33342 and propidium iodide were supplied from Roeche (Germany). All reagents were of analytical grade and used without further purification.

3. Methods

3.1. Preparation of PHBHHX nanoparticles

PHBHHX nanoparticles were prepared by the modified solvent evaporation technique and all the system parameters were changed to optimize the size and the size distribution of the nanoparticles. Briefly, 10 mg of PHBHHX was dissolved in 5 ml of dichloromethane to obtain 0.2% (w/v) PHBHHX solution as organic phase. Aqueous phase as a dispersion medium for the nanoparticle production were prepared by using 3 ml of Tween-80 and 50 ml of distilled water. Organic phase was added dropwise to the aqueous phase and homogenized using IKA T 125 Digital Ultra turrax homogenizer for 2 h. During the homogenization, mechanical stirrer was also used to get circular motion for the solution and to provide spherical shaped nanoparticles. The formed PHBHHX nanoparticles were recovered by centrifugation at 12,000 rpm for 30 min followed by washing with distilled water several times and lyophilized. For the cell culture studies, nanoparticles were sterilized under UV light for 30 min and later filtered with microfilter including 0.45 μm pore size.

To prepare nanoparticles loaded with drug, PHBHHX and Etoposide were completely dissolved in dichloromethane at different polymer/drug weight ratios (i.e., 1/0.5, 1/0.25 and 1/0.125) in the initial step of nanoparticle preparation procedure, followed by the same sequence as above. For the cell culture studies, nanoparticles were sterilized under UV light for 30 min and later filtered with microfilter including 0.45 μm pore size.

3.2. Immobilization of folic acid on PHBHHX nanoparticles

In this study; folic acid was covalently conjugated with PHBHHX nanoparticles in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) by the well-established carbodiimide method. EDAC was first reacted with carboxylic acid groups of PHBHHX which exist in the two ends of each polymer chain to form *o*-acrylisourea derivative. Then it was reacted with amine group of folic acid to yield amide bond between PHBHHX and folic acid, and leave the isourea derivative as a byproduct (Lin et al., 2004; Kavaz et al., 2010). Typically, folic acid (i.e., 250, 500, 750 and 1000 μg/mL) was dissolved in 1 ml of water by adjusting pH to weak alkaline. PHBHHX nanoparticle-folate conjugates were obtained by adding folate solution (2.5%, 5%, 7.5%, 10% w/w) into the activated 10.0 ml of PHBHHX nanoparticle suspension (1.0 mg/ml) corresponding to a PHBHHX:EDAC weight ratio of 1:1 and 2 h of activation time. The mixture was stirred at room