Synthesis and characterization of microbial poly3-hydroxybutyric acid nanocarrier for curcumin as an antibreast cancer agent

Mostafa A. El-Abd, Mona M. Agwa

Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Giza, Egypt

Correspondence to Dr. Mostafa A. El-Abd, Department of Chemistry of Natural and Microbial Products, Lab 174, National Research Centre, Dokki 12311, Giza, Egypt. Tel: +20 238 339 394; fax: +20 333 70931; E-mail: mostafaelabd77@yahoo.ca

Received: 16 February 2020
Revised: 28 March 2020
Accepted: 2 June 2020
Published: 24 August 2020

Egyptian Pharmaceutical Journal 2020, 19:266–273

Background and objective
In a previous study, the authors produced, optimized, characterized, and purified the poly3-hydroxybutyric acid (PHB) using the locally isolated *Bacillus flexus* bacterial strain. The development of a biodegradable drug carrier as an efficient delivery system has received great interest over the past few decades. The objective of this study was to produce a nano-PHB carrier for curcumin to be more effective in tumor fighting.

Materials and methods
PHB was produced on the optimized medium by *B. flexus* strain. The nano-PHB form was produced using the nanoprecipitation technique. The size, shape, and characteristics of loaded curcumin nano-PHB particles were performed using zeta-potential, scanning electron microscopy, and Fourier transform infrared spectroscopy techniques. The antitumor effect of the nano-PHB loaded curcumin was performed using human breast adenocarcinoma MCF-7 cell line.

Results and conclusion
In this study, a naturally developed, biodegradable, and biocompatible nanosized carrier for curcumin-targeted delivery in breast-cancer cells with higher encapsulation efficiency (95.5%) was formulated. The size range of both free PHB and curcumin-loaded PHB was 237 and 260 nm respectively. The nanoparticles exhibited a spherical shape with no aggregation which is confirmed by electron microscopy, indicating a higher colloidal stability. The curcumin-loaded PHB nanocarrier showed a sustained drug release behavior. In-vitro anticancer assays showed the superiority of curcumin-loaded PHB nanocarrier over free curcumin for fighting breast cancer. These results show that the PHB biopolymer acts as an efficient carrier vehicle for the curcumin.

Keywords:
Bacillus flexus, breast cancer, curcumin, poly3-hydroxybutyric acid

Egypt Pharmaceut J 19:266–273

1687-4315

Introduction

Breast cancer is one of the most common and lethal types of cancer in women worldwide, so it is considered to be the primary threat to women's health [1]. However, there is almost control for the disease by emergence of novel and new therapeutic products; the probability of relapse during disease progression is observed [2,3]. Multidrug resistance is the most common obstacle which leads to failure of many forms of breast cancer treatment [4]. Besides, cancer patients treated with available chemotherapeutic drugs usually suffer from sever adverse effects including vomiting, dysphoria, nausea, and sever toxicity [5]. So, there is an urgent need to discover new, safe, effective, evergreen, and appropriate strategies for delivering anticancer drugs with restricted side effects and management in the treatment of breast cancer [6]. Nanomedicine is considered one of these hopeful new therapeutic strategies. Nanomedicine refers to the biomedical application of materials having a size of between 1 and 1000 nm, which modulate both the selectivity and bioavailability of chemotherapeutic drugs [7]. The application of biodegradable polymers for administration and delivery of therapeutic agents has dramatically increased in recent years especially in the field of sustained drug delivery system [8]. They can be classified to either natural or synthetic polymers. Synthetic polymers displayed better advantages than the natural one as they can be easily designed to give a broad range of desirable features, but their toxicity limited their application. Natural polymers are considered safer and less toxic than synthetic ones; so, they recorded great advantages as drug carriers [9]. In this direction, a natural polyhydroxyalkanoate (a polymer of ester group), namely poly3-hydroxybutyrate (PHB), synthesized from renewable sources by various microorganisms in response to excess carbon and

nutrient limiting conditions displays considerable interest [10]. Numerous medical studies have reported the use of PHB as implants for tissue engineering or organs with no rejection as it is highly biocompatible and biodegradable [11,12]. Moreover, no secondary reactions lead to tissue necrosis or acute inflammation have been reported in tissue adjusted to PHB implants [13]. So, PHB has massive applications in medicine especially in the field of drug delivery for obtaining biomedically micro-carriers and nanocarriers as it did not induce immunogenic response *in vivo* [14].

The main disadvantage in cancer treatment is the lack of selectivity to tumor cells which leads to harmful side effects to noncancerous or normal cells. So, encouraging the uptake selectivity in the tumor site by targeted drug delivery is critical [15]. Overexpression of certain receptors on the surface of cancer cells (e.g. hydroxycarboxylic acid receptors) can be exploited for this purpose. Hydroxycarboxylic acid receptors on the cell surface is a good target for PHB nanocarriers which induce a highly cellular uptake for drugs loaded with PHB [16].

In many cases, cancer cells have been found to develop resistance over time toward chemotherapy, which led scientists to search for another treatments [17]. Natural products such as curcumin is considered as alternative to chemotherapy due to its potent anticancer activity and minimal side effects even at high doses[18]. Previous researches have reported that curcumin has various therapeutic activity including anti-inflammatory, anticancer, antibacterial, antioxidant, antiangiogenesis, and wound-healing properties [19,20]. Despite all these advantages, the clinical use of curcumin is very limited due to its poor solubility that leads to poor absorption, rapid metabolism by the liver, and fast elimination from the systemic circulation which restricted its administration as a chemotherapeutic drug. Therefore, a novel curcumin-targeted nanoformulation is critical to improve its delivery and to overcome the low curative effects [21].

In this study, we reported for the first time the fabrication of PHB nanocarrier loaded with curcumin anticancer drug for the treatment of breast cancer by targeting hydroxycarboxylic acid receptors expressed on the surface of breast cancer cells. PHB was first isolated from *Bacillus flexus* and then characterized. PHB nanocarrier was fabricated by an emulsification method with encapsulated curcumin and its antitumor efficacy was assessed *in vitro*. The size, morphology, release profile, and curcumin encapsulation were explored.

Materials and methods

Isolation, purification, and characterization of PHB from *Bacillus flexus* bacteria

These were done according to the methods mentioned by Zaghary *et al.* [22] and Singh and Avupati [23]. Briefly, PHB was isolated from the local strain *B. flexus* using viable colony-staining method using Nile red stain. Characterization of PHB was done using H+ and C13 NMR, GC, and Fourier transform infrared spectroscopy techniques. The experimental research that is reported in this manuscript has been performed with the approval of an appropriate ethics committee. No experiments had done on humans.

Preparation of curcumin-loaded PHB nanocarrier and its characterization

Ultraviolet spectrophotometric assay of curcumin

A stock standard solution of curcumin (Sigma Scientific Services Co., St. Louis, Missouri, USA) was prepared over the range of 0.1–0.6 mg% (w/v) in ethanol for λ_{max} detection by subsequent dilutions [22,23].

Preparation and optimization of curcumin-loaded PHB nanocarriers

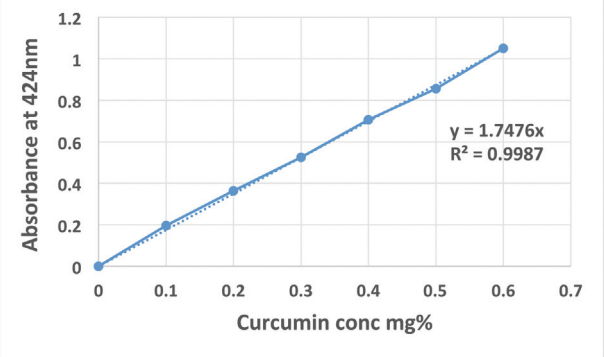
PHB-curcumin nanoparticles were prepared by the nanoprecipitation technique (Fig. 1) according to the method of Shakeri *et al.* [14] with some modifications. Briefly, PHB was dissolved in 1% PVA, mixed with curcumin and injected in a dropwise manner to distilled water along with Tween-80, and then the mixture was subjected to stirring for 5 h and then lyophilized.

Physicochemical characterization of nano-formulations

Process yield

The collected lyophilized powder was weighted and the yield was calculated by dividing these quantities by the total mass introduced in the preparation of nano-

Figure 1



Curcumin-loaded nanopoly3-hydroxybutyric acid formation using the nanoprecipitation technique.

formulation submitted to drying [24].

$$\text{Lyopholizationyield} = \frac{\text{Theweightofthefreezedriedpowdercollected}}{\text{Thetotalinitialmassofsolidsinthepreparationsubmittedtofreezedrying}}$$

Encapsulation efficiency and loading capacity

An aliquot of accurately weighed 25 mg of each batch of the freeze-dried nanoparticles was completely dissolved in 25 ml of ethanol and subjected to vigorous sonication to liberate any bound drugs. This solution was filtered through a 0.22 μm membrane filter and the amount of curcumin was assayed by UV at 424 nm depending on the previously constructed calibration curve. An equal weight of unloaded PHB nanoparticles was treated in the same manner to be used as a blank solution. The percentage loading capacity (%LC) and incorporation efficiency (%IE) for each formula were calculated using the equations of Li *et al.* [25] as follows:

$$\%\text{LC} = \frac{\text{Massofdruginnanoparticles}}{\text{Massofnanoparticlesrecovered}} \times 100$$

$$\%\text{IE} = \frac{\text{Massofdruginnanoparticles}}{\text{Massofdrugusedinformulation}} \times 100$$

Fourier transform infrared spectroscopy

The PHB nanoparticle functional groups were identified using the different modes of vibrations encountered with a spectrum ranging from 4000 to 400 cm^{-1} IR Affinity (Shimadzu, 3 Kanda-Nishikicho 1-chome, Chiyoda-ku, Tokyo, Japan) in transmission mode [26]. Then, the graph was plotted against wavelength and transmission rate.

Scanning electron microscopy

Morphological analysis of the prepared PHB nanoparticles was evaluated using a scanning electron microscope (SEM) (JSM-6700F; JEOL, 11 Dearborn Road Peabody, MA, USA). The dried powder samples were fixed on aluminum stubs using a double-sided adhesive tape and coated with gold at 2 mA for 3 min through sputter coating under an air atmosphere, which made the specimen electrically conductive. A SEM with a secondary electron detector was used to obtain digital images of the samples at an accelerating voltage of 10 kV. The geometric diameter (dG) was determined by size analysis of the SEM image of the powders using ImageJ software (NIH; 11 Dearborn Road Peabody, USA) with a minimum of 300 particle counts.

Particle size and zeta potential

The mean particle size and the particle distribution were determined using light scattering equipment (Zetasizer 3000 HSA, Malvern, and ALV-5000E, ALV). The lyophilized samples were diluted with double-distilled water without filtration.

In-vitro drug release

The dialysis membrane method was used to investigate the in-vitro release of drugs from the drug-loaded nanocarriers as compared with in-vitro release of free drug solution. A certain weight from the lyophilized formulation containing certain milligrams of curcumin was transferred into the dialysis bag with a molecular size cutoff of 12–14 kDa. The bags were suspended in 50 ml of pH 7.4 phosphate-buffered solution containing 0.5% (w/v) Tween-80 to maintain sink conditions and maintained at 37±0.5°C in a shaking water bath at 100 rpm. At designated time intervals, 1 ml samples of the dialysis medium was taken followed by compensation with the same volume of fresh release medium. All samples were run in triplicates and filtered through a 0.45 μm membrane filter, and the amount of curcumin released was analyzed by a UV spectrophotometer at 424 nm [27].

Cytotoxic effects on human cell lines

Cell viability was assessed by mitochondrial-dependent reduction of yellow MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to purple formazan [28].

Cells were suspended in DMEM medium for MCF-7, 1% antibiotic–antimycotic mixture (10 000 U/ml Potassium Penicillin, 10 000 μg/ml streptomycin sulfate and 25 μg/ml Amphotericin B), and 1% l-glutamine at 37°C under 5% CO_2.

Cells were batch cultured for 10 days, then seeded at a concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37°C for 24 h under 5% CO_2 using a water jacketed carbon dioxide incubator (TC2323; Sheldon, Cornelius, Oregon, USA). Media was aspirated; fresh medium (without serum) was added; and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentration of (100-50-25-12.5-6.25-3.125-0.78 and 1.56 μg/ml). After 48 h of incubation, medium was aspirated, 40ul MTT salt (2.5 μg/ml) was added to each well, and incubated for further 4 h at 37°C

under 5% CO_2. To stop the reaction and to dissolve the formed crystals, 200 µl of 10% SDS in deionized water was added to each well and incubated overnight at 37°C. A positive control which composed of 100 µg/ml was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions [29].

The anticancer activities of the prepared curcumin-loaded PHB nanocarrier, free curcumin, and blank PHB nanocarrier were carried out on human breast adenocarcinoma MCF-7 cell line purchased from the American Type Culture Collection (USA).

The absorbance was then measured using a microplate multiwell reader (model 3350; Bio-Rad Laboratories Inc., Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm.

Results and discussion

Spectrophotometric assay of curcumin

The principal peak obtained with the standard solution was at 424 nm which was selected as the λ_{max} of the drug. A good linearity was shown by the calibration curve obtained from 0.1 to 0.6 mg% curcumin concentration as shown in Fig. 2. A coefficient of determination (r^2) of 0.9987 was obtained from the linear regression analysis of data (absorbance vs concentration).

Physicochemical characterization of nano-formulations

Process yield

Freeze-drying (lyophilization) is a drying method that applied for the long-term preservation of heat-sensitive biological materials such as DNA, enzymes, and proteins and pharmaceutical products (antibiotics) to improve the stability of formulations [30]. Freeze-dried nanoparticles should have particular desirable features, which includes easy reconstitution by water addition, long-term stability of the sample at room temperature, low particle size distribution of nanoparticle suspensions, reduction in weight, unchanged efficiency of the encapsulated drug, and the possibility of easy sterile handling [31].

Pleasant observation of PHB during the freeze-drying process was its capability to perform as a stabilizing agent, which evades the need of drying adjuvants or it may be minimal, so leading to a higher drug loading efficacy of drug-loaded nanocarriers.

In this study, the freeze-drying technique was used for the conversion of nanoparticle suspension into redispersible solid nanoparticles. The process enabled the collection of high-yield powders (96.5%). Furthermore, easily reconstituted freeze-dried nanoparticles were obtained that exhibit a nano-range particle size with no coagula observed in solution after reconstitution.

Fourier transform infrared spectroscopy

Strong peak at 1627 cm^{-1} shows the presence of C<td: glyph name=″dbnd″/>O which is the characteristic of PHB and peak at 2925 cm^{-1} shows the presence of alkyl C-H bond. The presence of curcumin in loaded samples was confirmed by the presence of a peak at 3435 cm^{-1} for the presence of OH bond, whereas 963 cm–1 was indicating the =C-H bond. 1508–1426 cm^{-1} shows the C-C bond and 1026 cm^{-1} shows the presence of C-O bond. The C–O–H bond of curcumin was found at 1275 cm^{-1}. C–O of curcumin was found between 1153 cm^{-1} (Fig. 3) [32–35].

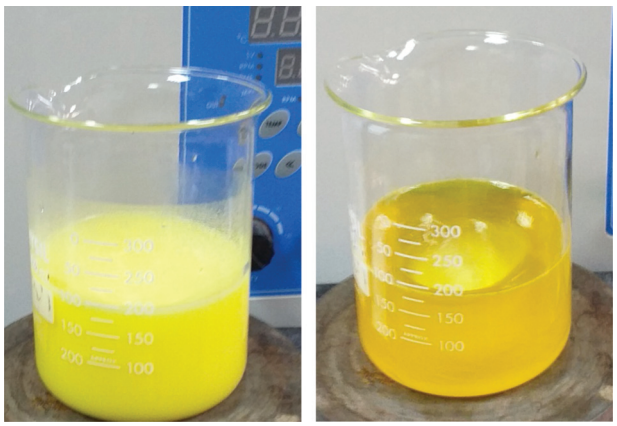
Particle size and zeta potential

The particle size, size distribution of PHB nanocarrier, and PHB nanocarrier loaded with curcumin were 200 and 230 nm, respectively, and are presented in Figs 4 and 5. These results agreed with other previous findings showing that the size of PHB nanospheres varied from 128 to 320 nm [36]. Other results showed that the size of PHB nanocarrier loaded with Doxorubicin was in the range between 250 and 300 nm [37].

Morphological analysis by SEM

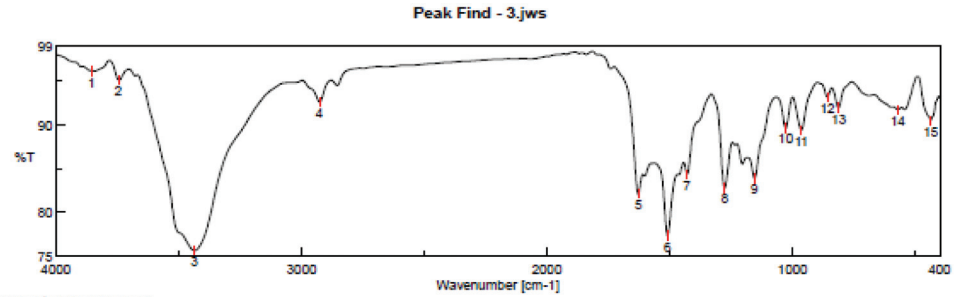
Figure 6 shows the morphology of the loaded curcumin on PHB which appear to be rounded to oval in shape with smooth and regular surface.This result is consistent with what has already been published which showed that the SEM analysis of the

Figure 2



Ultraviolet calibration of curcumin at λ_{max}424 nm.

Figure 3

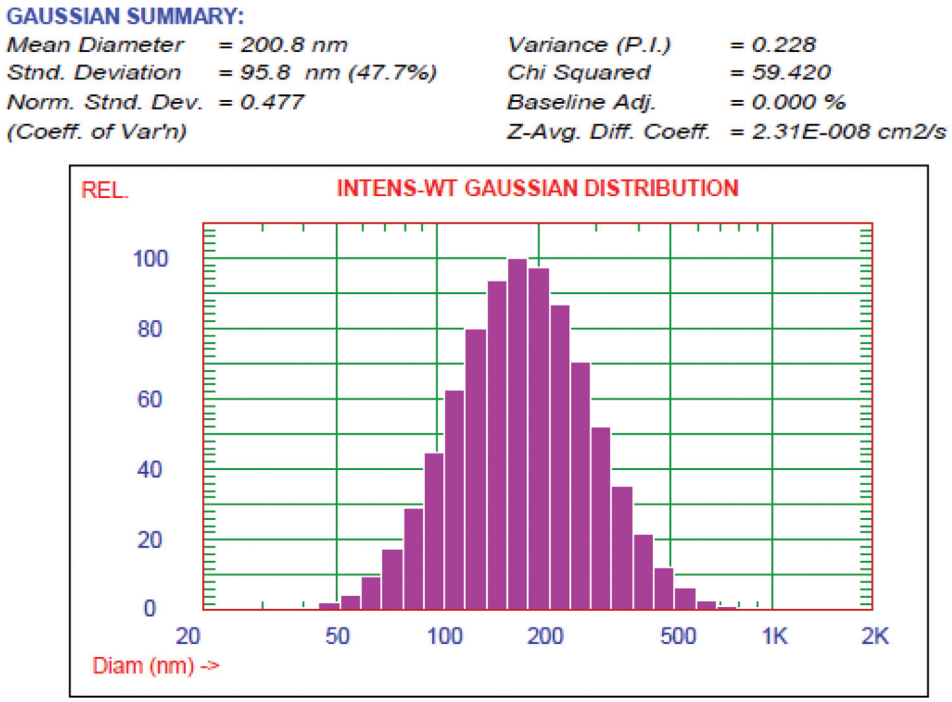


[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3854.04	96.1049	2	3745.08	95.1188	3	3435.56	75.6243	4	2925.48	92.6057
5	1627.63	82.0536	6	1508.06	77.3184	7	1428.03	84.346	8	1275.68	82.7508
9	1153.22	83.8331	10	1026.91	89.6098	11	963.269	89.3808	12	857.204	93.1459
13	811.885	91.9356	14	570.826	91.7168	15	435.834	90.4911			

Fourier transform infrared spectroscopy of curcumin-loaded nanopoly3-hydroxybutyric acid.

Figure 4



Particle size and zeta potential of nanopoly3-hydroxybutyric acid using a zetasizer.

prepared PHB nanocarriers showed a spherical and smooth surface [10,11,36–38].

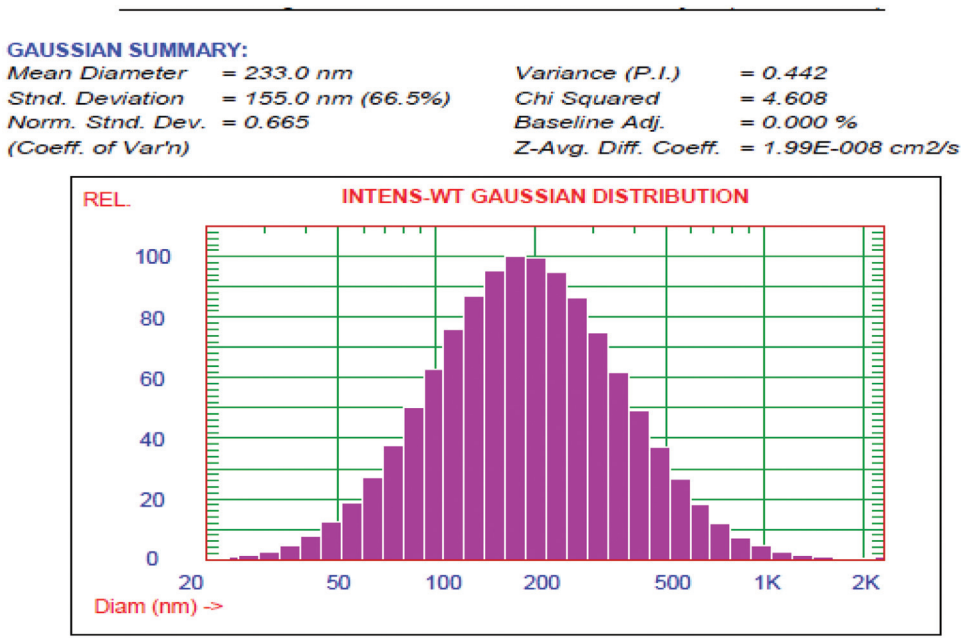
Encapsulation efficiency and loading capacity

In this study, the analysis of the curcumin content of the lyophilized PHB nanocarrier loaded with curcumin indicated that the drug incorporation efficiency (%IE) was 95.5% and the drug loading capacity (%LC) was 18.75%. Vidal *et al.* [39] showed a high incorporation efficiency for quercetin loaded polyhydroxybutyrate-*co*-hydroxyvalerate (51%). The higher encapsulation efficiency (61%) was also recorded for Silymarin-loaded PHB nanoparticles [38].

In-vitro drug release

In-vitro curcumin release pattern from lyophilized PHB nanocarriers loaded with curcumin showed a sustain and biphasic release profile, which is characterized by an initial relatively fast release during the first 8 h (55%), followed by continuous sustained release of up to 48 h (65%) compared with free drug 100% after 3 h as shown in Fig. 7. A sustained release behavior was previously recorded for PHB-loaded quercetin [39] and doxorubicin [37]. Also, the release behavior of Silymarin-loaded PHB nanoparticles in PBS showed a sustained release behavior during 48 h [38].

Figure 5



Particle size and zeta potential of curcumin-loaded nanopoly3-hydroxybutyric acid using a zetasizer.

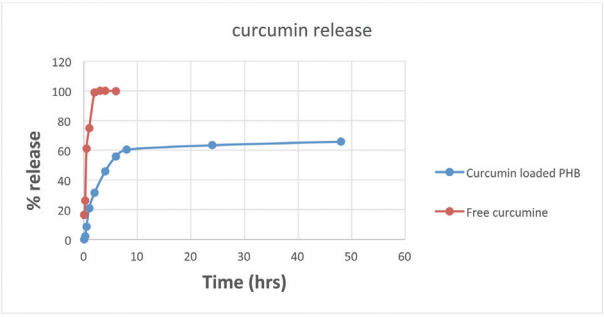
Figure 6

Scanning electron microscopy of curcumin-loaded nanopoly3-hydroxybutyric acid.

In-vitro antitumor activity

Cytotoxic studies were conducted to examine the potential effects of the fabricated nanocarrier for enhancing the activity of curcumin. Cytotoxic potency of free curcumin, PHB nanocarrier, and PHB nanocarrier loaded with curcumin were investigated against human breast cancer (MCF-7) cells after 48 h incubation. The IC_{50} of both free curcumin and PHB nanocarrier loaded with curcumin at 48 h were found to be 83.3±5.9 and 44.8±3.4 µg/ml, respectively. On the other hand, no anticancer activity was recorded for free PHB nanocarrier. The authors attributed the higher anticancer activity for PHB nanocarrier loaded with curcumin than free curcumin to the higher solubility of the prepared nanocarrier compared with the free drug

Figure 7



In-vitro curcumin release profile from poly3-hydroxybutyric acid nanoparticles.

solution which results in higher concentrations around the cells and subsequent higher cytotoxicity [40]. Also, the PVA-PHB core-shell particles were developed as suitable carriers to protect drug by degradation to increase the required therapeutic concentration. Thus, nanoparticles are expected to reach, accumulate into the tumor cells, and release the entrapped curcumin [10].

Conclusion

In this study, curcumin was successfully loaded on PHB nanoparticles with an encapsulation efficiency of 95.5%. The size of curcumin-loaded PHB was 260 nm. The curcumin-loaded PHB showed a sustained release profile. The PHB nanocarrier loaded with curcumin was found to be more effective in breast cancer. Further studies have to be conducted for other tumor types where both PHB and curcumin are natural products without the harmful side effects of the chemotherapy in tumor fighting.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1 Lei M, Sha S, Wang X, Wang J, Du X, Miao H, *et al.* Co-delivery of paclitaxel and gemcitabine via a self-assembling nanoparticle for targeted treatment of breast cancer. RSC Adv 2019; 9:5512–5520.

2 Taylor C, Correa C, Duane FK, Aznar MC, Anderson SJ, Bergh J, *et al.* Estimating the risks of breast cancer radiotherapy: evidence from modern radiation doses to the lungs and heart and from previous randomized trials. J Clin Oncol 2017; 35:1641–1651.

3 Kommineni N, Mahira S, Domb AJ, Khan W. Cabazitaxel-loaded nanocarriers for cancer therapy with reduced side effects. Pharmaceutics 2019; 11:1–22.

4 Ford JM, Hait WN. Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol Rev 1990; 42:155–199.

5 El-Far SW, Helmy MW, Khattab SN, Bekhit AA, Hussein AA, Elzoghby AO. Folate conjugated vs PEGylated phytosomal casein nanocarriers for codelivery of fungal-and herbal-derived anticancer drugs. Nanomedicine 2018; 13:1463–1480.

6 Pareja F, Marchiò C, Geyer FC, Weigelt B, Reis-Filho JS. Breast cancer heterogeneity: roles in tumorigenesis and therapeutic implications. Curr Breast Cancer Rep 2017; 9:34–44.

7 García-Pinel B, Porras-Alcalá C, Ortega-Rodríguez A, Sarabia F, Prados J, Melguizo C, López-Romero JM. Lipid-based nanoparticles: application and recent advances in cancer treatment. Nanomaterials 2019; 9:1–23.

8 Lu Y, Chen S. Micro and nano-fabrication of biodegradable polymers for drug delivery. Adv Drug Deliv Rev 2004; 56:1621–1633.

9 Elzoghby AO, El-Fotoh WSA, Elgindy NA. Casein-based formulations as promising controlled release drug delivery systems. J Control Release 2011; 153:206–216.

10 Radu IC, Hudita A, Zaharia C, Galateanu B, Iovu H, Tanasa E, *et al.* Poly (3-hydroxybutyrate-CO-3-hydroxyvalerate) PHBHV biocompatible nanocarriers for 5-FU delivery targeting colorectal cancer. Drug Deliv 2019; 26:318–327.

11 Pachiyappan S, Selvanantham DS, Kuppa SS, Chandrasekaran S, Samrot AV. Surfactant-mediated synthesis of polyhydroxybutyrate (PHB) nanoparticles for sustained drug delivery. IET Nanobiotechnol 2019; 13:416–427.

12 Shrivastav A, Kim HY, Kim YR. Advances in the applications of polyhydroxyalkanoate nanoparticles for novel drug delivery system. Biomed Res Int 2013; 2013:1–12.

13 Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol 2008; 20:86–100.

14 Shakeri F, Shakeri S, Hojjatoleslami M. Preparation and characterization of carvacrol loaded polyhydroxybutyrate nanoparticles by nanoprecipitation and dialysis methods. J Food Sci 2014; 79:697–705.

15 Mishra N, Pant P, Porwal A, Jaiswal J, Aquib M. Targeted drug delivery: a review. Am J Pharm Tech Res 2016; 6:2249–3387.

16 Stäubert C, Broom OJ, Nordström A. Hydroxycarboxylic acid receptors are essential for breast cancer cells to control their lipid/fatty acid metabolism. Oncotarget 2015; 6:1–15.

17 Yallapu M, Jaggi M, Chauhan S. Curcumin nanomedicine: a road to cancer therapeutics. Curr Pharm Des 2013; 19:1994–2010.

18 Wong KE, Ngai SC, Chan KG, Lee LH, Goh BH, Chuah LH. Curcumin nanoformulations for colorectal cancer: a review. Front Pharmacol 2019; 10:1–17.

19 Krausz AE, Adler BL, Cabral V, Navati M, Doerner J, Charafeddine RA, *et al.* Curcumin-encapsulated nanoparticles as innovative antimicrobial and wound healing agent. Nanomed Nanotechnol Biol Med 2015; 11:195–206.

20 Vallianou NG, Evangelopoulos A, Schizas N, Kazazis C. Potential anticancer properties and mechanisms of action of curcumin. Anticancer Res 2015; 35:645–651.

21 Kundu M, Sadhukhan P, Ghosh N, Chatterjee S, Manna P, Das J, Sil PC. pH-responsive and targeted delivery of curcumin via phenylboronic acid-functionalized ZnO nanoparticles for breast cancer therapy. J Adv Res 2019; 18:161–172.

22 Zaghary W, Hanna E, Zanoun M, Abdallah N, Sakr M. Curcumin: analysis and stability. J Adv Pharm Res 2019; 3:47–58.

23 Singh A, Avupati VR. Development and validation of UV-spectrophotometric method for the estimation of curcumin in standardised polyherbal formulations. J Young Pharm 2017; 9:491–495.

24 Oyarzun-Ampuero FA, Rivera-Rodríguez GR, Alonso MJ, Torres D. Hyaluronan nanocapsules as a new vehicle for intracellular drug delivery. Eur J Pharm Sci 2013; 49:483–490.

25 Li C, Li X, Li S, Weng Y, Wang K. Development and validation of a method for determination of encapsulation efficiency of CPT-11/DSPE-mPEG2000 nanoparticles. Med Chem (Los Angeles) 2016; 6:345–348.

26 Dipankar C, Murugan S. The green synthesis, characterization and evaluation of the biological activities of silver nanoparticles synthesized from Iresine herbstii leaf aqueous extracts. Colloids Surfaces B: Biointerfac 2012; 98:112–119.

27 Pooja D, Kulhari H, Tunki L, Chinde S, Kuncha M, Grover P, *et al.* Nanomedicines for targeted delivery of etoposide to non-small cell lung cancer using transferrin functionalized nanoparticles. RSC Adv 2015; 5:49122–49131.

28 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65:55–63.

29 Thabre MI, Hughes RD, Mcfarlane IG. Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. J Pharm Pharmacol 1997; 49:1132–1135.

30 Kusakabe H, Kamiguchi Y. Chromosomal integrity of freeze-dried mouse spermatozoa after 137Cs γ-ray irradiation. Mut Res Fundamental Mol Mech Mutagen 2004; 556:163–168.

31 Ciurzyńska A, Lenart A. Freeze-drying-application in food processing and biotechnology – a review. Pol J Food Nutr Sci 2011; 61:165–171.

32 Hong K, Sun S, Tian W, Chen G, Huang W. A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. Appl Microbiol Biotechnol 1999; 51:523–526.

33 Senthilvelan N, Rajarajan G, Jegatheesan A, Sivakumar S, Elanchezhiyan J. Growth and spectroscopic characterization of urea sulfamic acid crystal: a second-order nonlinear material. Rasayan J Chem 2017; 10:218–225.

34 Wiley J. Votre tour!: Intermediate French/Activities Manual. France, Paris: John Wiley; 2006.

35 Senthilkumar P, Dawn S, Samanvitha KS, Kumar SS, Kumar GN, Samrot AV. Optimization and characterization of poly [R] hydroxyalkanoate of *Pseudomonas aeruginosa* SU-1 to utilize in nanoparticle synthesis for curcumin delivery. Biocatal Agric Biotechnol 2017; 12: 292-298.

36 Abid S, Raza Z, Rehman A. Synthesis of poly (3-hydroxybutyrate) nanospheres and deposition thereof into porous thin film. Mater Res Express 2016; 3:105042.

37 Sasikumar P, Ayyasamy P. Design and characterization of poly-hydroxy butyric acid (PHB) based polymeric nanoparticles for controlled release of doxorubicin for cancer treatment. Int J Curr Microbiol App Sci 2015; 4:311–317.

38 Radu I, Hudita A, Zaharia C, Stanescu PO, Vasile E, Iovu H, *et al.* Poly (hydroxybutyrate-co-hydroxyvalerate)(PHBHV) nanocarriers for silymarin release as adjuvant therapy in colo-rectal cancer. Front Pharmacol 2017; 8:1–12.

39 Lightfoot Vidal S, Rojas C, Bouza Padín R, Pérez Rivera M, Haensgen A, González M, Rodríguez-Llamazares S. Synthesis and characterization of polyhydroxybutyrate-co-hydroxyvalerate nanoparticles for encapsulation of quercetin. J Bioact Compat Polym 2016; 31:439–452.

40 Wang J, Zhu R, Sun X, Zhu Y, Liu H, Wang S. Intracellular uptake of etoposide-loaded solid lipid nanoparticles induces an enhancing inhibitory effect on gastric cancer through mitochondria-mediated apoptosis pathway. Int J Nanomed 2014; 9:3987–3998.