Characterization of Polyhydroxybutyrate Nanoparticles

Jenan A. Ghafil*, May T. Flieh
College of Science, Department of Biology, University of Baghdad, Baghdad, Iraq

Received: 2/3/2021Accepted: 1/5/2021

Abstract
Preparation of nanoparticles is one of the important ways to increase the biological effectiveness of materials. There are several methods to prepare the polyhydroxybutyrate (PHB) nanoparticles. Here, a new method is used based on exposing PHB to ultrasound waves under variable pH conditions. In the present study, PHB was added to distilled water and pH was adjusted to 4 by HCl (1 N). The suspension was exposed to ultrasound waves at 4500 kh for 25 seconds. Then, pH was readjusted to 10 by NaOH (1N) and the mixture was incubated for 2 h at 21 °C. Finally, the pH was adjusted to 7 by HCl (1 N) and the mixture was incubated at 21 °C for 18 h. The characterization of the prepared nanoparticles was achieved by using atomic force microscopy (AFM), Fourier-transform infrared spectroscopy (FTIR), ultraviolet (UV) spectrophotometer, X-ray powder diffraction (XRD), and scanning electron microscopy (SEM). The results demonstrated the formation of nanoparticles, especially after examinations by SEM and AFM, which showed that the diameter of particles was between 22.9 and 73.95. The present study confirmed that the method of exposing PHB to gradient pH and high levels of ultrasonic waves could produce PHB nanoparticles.

Keywords: Polyhydroxybutyrate (PHB), PHB nanoparticles, pH gradient method, Ultrasonic waves.
Introduction

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoate polymer belonging to the polyesters class that has the area of interest as bio-derived and biodegradable plastics. The poly-3-hydroxybutyrate (P3HB) form of PHB is probably the most common type of polyhydroxyalkanoate, but other polymers of this class are produced by a variety of organisms. These include poly-4-hydroxybutyrate (P4HB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH), polyhydroxyoctanoate (PHO), and their copolymers [1]. PHA is one of many biopolymers of linear-chain ester groups that are produced as a carbon source and energy reserve in bacteria during physiologically stressful conditions. They exhibit good physiochemical properties that are exploited for many biomedical applications. PHB is the first to be found under the category of four carbon chained homopolymers. It is universally known as an alternative for plastics. PHB is being used as an implantation material, biofuel, etc. [1].

PHB is a material which also generates interest by having different approaches for synthesis. There are three routes of obtaining PHB, the first being by means of polymerization synthesis [2]. Nanoparticles (NPs) are commonly used as antimicrobial agents that affect microbial growth in different ways, especially biofilm formation [3] and occasionally by stimulating the innate immune response to pathogens in vivo [4]. Nanoparticles are ultrafine particles that are ranging between 1 and 100 nm in size. The chemical nature of nanoparticles may be metallic or polymeric. Polymeric nanoparticles may be synthetic or natural and exhibit nano-sized colloidal structures [5]. Based on the preparation method of nanoparticles, drugs can be loaded into or onto the surface. In nano capsules, the drug is entrapped by the polymeric surface surrounding the molecule. Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Nanoparticles (NPs) are methods used to detect

In the present study, we used a new method of NPs synthesis that is based on exposing the PHB to ultrasound under different pH values.

Materials and methods

Materials

PHB was purchased from Sigma-Aldrich, USA. PHB used in the present study was derived from microbial fermentation.

Synthesis of PHB Nanoparticles-

One gram of PHB was added to 50 ml of distilled water and pH was adjusted to 4 by HCl (1 N). The mixture was placed in an ultrasonic path at 4500 kh for 25 seconds. The pH was readjusted to 10 by NaOH (1N). The mixture was mixed by a magnetic stirrer for 2 hrs. at 21 °C. The mixture was incubated at 21 °C for 18 hrs. and then the pH was readjusted to 7 by HCl (1 N). The characterization of the prepared nanoparticles was achieved by using atomic force microscopy (AFM), Fourier-transform infrared spectroscopy (FTIR), ultraviolet (UV) spectrophotometer, X-ray powder diffraction (XRD), and scanning electron microscopy (SEM) [7].

Characterization of PHB Nanoparticles

Different standard methods were followed to judge whether the prepared particles of PHB were PHB nanoparticles.
UV-Vis spectral analysis
PHB nanoparticles synthesis was confirmed by measuring the wavelength of the prepared mixture by UV-Vis spectrum at a resolution of 1 nm in 2 ml quartz cuvette with 1 cm path length. Scanning range for the samples was 190-1100 nm at a scan speed of 500 nm/min. A blank reference was used for the correction of the spectrophotometer. The UV-Vis absorption spectra of all the samples were recorded and numerical data were plotted [5].

Atomic force microscopy (AFM) analysis
Atomic force microscopy was used to analyze the prepared PHB nanoparticles. A thin film of the prepared PHB nanoparticles was deposited on a silica glass plate by dropping few drops of the mixture of PHB nanoparticles on the plate and allowing them to dry at room temperature in the dark. The deposited film glass plate was then scanned with the AFM [8].

Scanning electron microscope (SEM)
The main features of morphology and diameter of nanoparticles were characterized using SEM. The dried sample of PHB nanoparticles solution was sonicated with distilled water; few drops of the prepared sample were placed on a glass slide and allowed to dry. After that, a thin layer of platinum was coated to make the samples conductive [9].

X-ray diffraction
In this technique, a glass slide was prepared that contains PHB nanoparticles at a concentration of 20 mg/ml (several drops) to form a thin layer of PHB suspension with a thickness of 0.5 mm. After that, it was examined using an X-ray diffraction device by exposing to K and Cu rays over the model to be measured, at different angles (10° to 80°), with a wavelength of 1.5406 Å, a voltage of 40 kilovolts, and an amount of 2°/min [7].

Fourier-transform infrared spectroscopy (FTIR)
FTIR is a technique which is used to obtain infrared spectra of absorption, emission, and photoconductivity of solid, liquid, and gas. It was used to detect different functional groups in PHB. FTIR spectra are recorded between 4000 and 400 cm−1. For FTIR analysis, the polymer was dissolved in chloroform and layered on a NaCl crystal. After the evaporation of chloroform, the polymer film was subjected to FTIR. The spectrum of PHB showed peaks at 1724 cm−1 and 1279 cm−1, which correspond to specific rotations around carbon atoms. The peak at 1724 cm−1 corresponds to C–O stretch of the ester group present in the molecular chain of a highly ordered structure. The adsorption band at 1279 cm−1 corresponds to ester bonding [12].

Results
PHB nanoparticles preparation
UV-Vis spectral analysis
The PHB nanoparticles were examined with visible and UV spectrophotometers. The results showed that three peaks were raised. The absorbance values of the peaks were 2.967 and 0.595 at the wavelengths of 283 and 311.00, respectively (Figure 1).
Atomic force microscopy (AFM) analysis
In the present study, PHB nanoparticles that were prepared from PHB were examined under AFM apparatus to confirm the presence of PHB nanoparticles. The results demonstrated the existence of a number of nanoparticles with different diameters, with an average of 79 nanometers (Figures 2 and 3). In all examined samples, the nanoparticles were found to be spherical-shaped and aggregated, with smooth surfaces.

**Figure 1**-Absorbency of PHB nanoparticles under different wavelengths. The range of wavelength was 0000 to 0000 nm.

**Figure 2**-Atomic force microscopy (AFM) analysis showing the diameters of PHB nanoparticles.
Figure 3-Scanned picture under AFM apparatus showing the diameters of the prepared PHB nanoparticles.

**Scanning electron microscopy**

The PHB nanoparticles prepared in the present study were analyzed by SEM to determine their diameter and shape. The results showed clearly that the range of diameters of the PHB nanoparticles was from 15.5 to 34.8 nm, with an average of 22.9 nm. The data of size of particles were collected by using the Digimizer software. The present study showed that the prepared PHB typically formed nanoparticles with a very small size. These results confirmed that the method used in preparing PHB nanoparticles was very efficient (Figure 4a and b).

Figure 4-a and b. PHB nanoparticles picture taken by scanning electron microscope. The diameters of particles ranged from 34.8 to 15.5 nm with an average of 22.9 nm. The data were analyzed by Digimizer software.
X- Ray diffraction
The prepared PHB nanoparticles were examined by XRD and the results showed different peaks, but there were only four main peaks at 13.4, 17.2, 32 and 45.8 Å. The largest peak was seen at 45.8 Å (Figure 5).

![Figure 5](image)

**Figure 5** - The XRD patterns of PHB nanoparticles. The scale shows four main peaks.

Fourier-transform infrared spectroscopy
The prepared PHB nanoparticles were subjected to FTIR analysis, as shown in Figure 6. From the spectrum obtained, it is inferred that the bands at 3458.17 cm\(^{-1}\) and 3440.77 cm\(^{-1}\) correspond to OH (Hydroxyl) group, whereas the band at 1728.1 cm\(^{-1}\) represents C=O (Carbonyl) and COO (ester) groups. The band at 1371 cm\(^{-1}\) represents COH bond, whereas the band at 1288 cm\(^{-1}\) corresponds to CH. The results also showed asymmetrical stretching and bending vibration in the CH\(_3\) group stretch of bands ranging 1058.85 -1288.36 cm\(^{-1}\), which showed C-O bonding. The analyzed results are consistent with the reports of Arun *et al.* (2009) and Padermshoke *et al.* (2004), confirming that the isolated compound was PHB.

![Figure 6](image)

**Figure 6** - Fourier transform infrared spectroscopy spectral analysis of PHB nanoparticles in the 400–4000 cm\(^{-1}\) wave number region.
Discussion

There are several methods that were used by many investigators to prepare PHB nanoparticles [13, 14]. In the present study, a different method was used for the preparation; the method was dependent on exposing the PHB suspension to ultrasound at different pH values. Different tests (UV-Vis spectra analysis, AFM, SEM, XRD and FTIR) were performed to check the presence of PHB nanoparticles as an indicator of success of the method used in the present study.

The results of previous studies, which used UV-Vis spectra analysis to check the presence of PHB nanoparticles, showed that the peaks of PHB absorbency for PHB prepared from Azohydromonas australica DSM 1124 were found at 235 nm [15]. A similar result was found by Alarfaj et al. (2015) [16]. These results are similar to, but not the same as, those found in the present study in terms of the peak of absorbency, because the particles used in the present study are nanoparticles.

The size of PHB nanoparticles varies according to several factors, such as the method of preparation, source of PHB, and environment of PHB nanoparticles preparation. Senthilkumar et al. (2018) found that PHB nanoparticles prepared by the nanoprecipitation method with different solvent systems, i.e. Chloroform: DMSO (CD), Chloroform: Water (CW), Ethylacetate: DMSO (ED), and Ethylacetate: Water (EW), had different sizes that ranged 110 – 300 nm after examination by AFM [17]. Pachiyanpan et al. (2019) prepared PHB nanoparticles by following the nanoprecipitation method with different solvents and surfactant (Tween 80) concentrations. They found that the diameters of the nanoparticles, as examined by AFM, ranged from 60 to 300 nm [18].

SAM is one of the most important and accurate methods to determine the sizes and shapes of nanoparticles obtained through practical experiments. Most of previous studies that dealt with preparing nanoparticles relied on SAM to determine their success in obtaining nanoparticles, since, by using this method, the nanoparticles can be seen and their pictures can be acquired [15-18].

Previous studies also showed the results of the utilization of XRD to detect PHB nanoparticles. Liau et al. (2014) showed very small peaks at 2θ of 2.195, 2.275, and 2.325° with 40.20, 38.78, and 38.00’A interlayer spacing values, respectively. This increment of d-spacing indicates that polymer chains are intercalated into the clay layers to form intercalated type nanocomposites [19]. Mottin et al. (2016) examined the prepared PHB nanoparticles using an XRD apparatus and their results are slightly different from those of the present study because the nanoparticles they prepared are nanofiber particles [20]. The results of other previous studies were in agreement with those of the present study [21, 22].

When the prepared PHB nanoparticles were examined by FTIR, different peaks were seen that match those observed in other studies. Those prepared and purified PHB nanoparticles from several sources that according to the several reasons such as the source of PHB [17].

The present study showed that the PHB particles prepared by the selected method are typically nanoparticles. This leads to conclude that the method used in the present study is useful to prepare PHB nanoparticles.

References

[1] B. McAdam, B. Fournet, M. P. McDonald and M. Mojicevic "Production of Polyhydroxybutyrate (PHB) and Factors Impacting Its Chemical and Mechanical Characteristics" Polymers, vol. 12(12), no. 2, pp. 2908. 2020.
[2] D.S.W. Benoit, K.R. Sims and D. JrFraser, "Nanoparticles for Oral Biofilm Treatments" American Chemical Society, vol. 13, no.3, pp. 4869-4875. 2019.
[3] B.J. Swartzwelter, A.C. Fux, L. Johnson, E. Swart, S. Hofer, N. Hofstätter, M. Geppert, P. Italiani, D. Boraschi, A. Duschl and M. Himly, "The Impact of Nanoparticles on Innate
Immune Activation by Live Bacteria", *International Journal of Molecular Sciences*, vol. 21, no. 24, pp. 9695. 2019.

[4] Y. Wu, M. Wang, S. Luo, Y. Gu, D. Nie, Z. Xu, Y. Wu, M. Chen and X. Ge, "Comparative Toxic Effects of Manufactured Nanoparticles and Atmospheric Particulate Matter in Human Lung Epithelial Cells", *International journal of environmental research and public health*, vol. 18, no. 1, pp. 22.2020.

[5] G. Lin, Q. Zhang, X. Lin, D. Zhao, R. Jia, N. Gao, Z. Zuo, X. Xu and D. Liu, "Enhanced photoluminescence of galliumphosphate by surface plasmon resonances of metallic nanoparticles", *RSC Advances: an international journal to further the chemical sciences*, vol. 5, no. 1, pp. 48275–48280. 2015.

[6] P. Senthilkumar1, S. S. Dawn, C. Saipriya and V. Antony," Synthesis of polyhydroxybtyurate nanoparticles using surfactant for hydrophobic delivery", *Rasayan Journal of Chemistry*, vol. 7, no 26, pp. 6778-6786. 2018.

[7] S. Deng, M. R. Gigliobianco, R. Censi and Di. Martino," Polymeric Nanocapsules as Nanotechnological Alternative for Drug Delivery System: Current Status, Challenges and Opportunities", *Nanomaterials* (Basel, Switzerland), vol. 10, no. 3, pp. 847. 2020.

[8] W. Haiss, N.T. Thanh, J. Aveyard and D.G. Fernig," Determination of size and concentration of gold nanoparticles from UV-vis spectra", *Analytic Chemistry*, vol. 1, no.7, pp.9.2007.

[9] Y. Kikkawa, M. Fujita, T. Hiraishi, M. Yoshimoto and Y. Doi," Direct observation of poly(3-hydroxybutyrate) depolymerase adsorbed on polyester thin film by atomic force microscopy", *Biomacromolecules*, vol. 5, no. 5, pp.1642.2004.

[10] S. Krishnan, G.S. Chinnadurai and P. Perumal, " Polyhydroxybutyrate by Streptomyces sp.: Production and characterization", *International Journal of Biological Macromolecules*, vol. 104, no. 4, pp.1165-1171. 2017.

[11] H. Sato, R. Murakami and J. Zhang, "X-ray diffraction and infrared spectroscopy studies on crystal and lamellar structure and cho hydrogen bonding of biodegradable poly(hydroxyalkanoate)*, *Macromol*, vol. 14, no.1, pp. 408–415. 2006.

[12] R. Sindhu and P. Pandey ,"Microbial Poly-3-Hydroxybutyrate and Related Copolymers", *Industrial Biorefineries & White Biotechnology*, vol. 4, no.3, pp. 575-605, 2015

[13] S. Ram, V. Deepak, K. Kalishwaralal, J. Muniyandi and N. Gurunathan, " Synthesis of PHB nanoparticles from optimized medium utilizing dairy industrial waste using Brevibacterium casei SRKP2: a green chemistry approach", *Colloids Surf Biointerfaces*, vol.1, no. 7, pp. ,266-73. 2009.

[14] S.R. Pandian, V. Deepak, H. Nellaiah and K. Sundar," PEG-PHB-glutaminase nanoparticle inhibits cancer cell proliferation in vitro through glutamine deprivation*. In *In Vitro Cellular & Developmental Biology*, vol. 51, no. 4, pp. 372-80. 2014.

[15] S. Ahmad, A. Amir, M. Zafaryab, K. Osama and S.A. Faridi," Production and Characterization of Polyhydroxybutyrate Biopolymer from Azohydromonas Australica Using Sucrose as a Sole Carbon Source*. Journal of Microbial & Biochemical Technolog, vol. 9, no.3, pp. 082-086.2017.

[16] A. A. Alarfaj, M. Arshad, E. Sholkamy, N and M. A. Munusamy," Extraction and Characterization of Polyhydroxybutyrates (PHB) from Bacillus thuringiensis KSADL127 Isolated from Mangrove Environments of Saudi Arabia", *Brazilian Archives of Biology and Technology*, vol. 58, no.3, pp. 781-788. 2015.

[17] P. Senthilkumar, S.S. Dawn, C. Saipriya and A.V. Samrot, " Synthesis of polyhydroxybutyrate nanoparticles using surfactant (Span20) for hydrophobic drug delivery*. *Rasayan Journal of Chemistry*, vol. 11, no. 4, pp. 1686-1695. 2018.

[18] S. Pachiyappan, D.S. Selvanantham,S .S. Kuppa, , S. Chandrasekaran and A.V. Samrot, "Surfactant-mediated synthesis of polyhydroxybutyrate (PHB) nanoparticles for sustained drug delivery", *Nanobiotechnology*, vol. 13, no. 4, pp. 416 – 427, 2019.

[19] C.P. Liau, M.B. Ahmad, K. Shameli, W.M.Z. Yunus, W. Ibrahim, N.A. Zainuddin, N.N and Y.Y. Then, "Preparation and Characterization of Polyhydroxybutyrate/Polycaprolactone Nanocomposites", *The Scientific World Journal*, vol. 9, no.2, pp. 39, 2014.

[20] A.C. Mottin, E. Ayres, R.L. Oréfice and J.J.D. Câmara, " What Changes in Poly(3-Hydroxybutyrate) (PHB) When Processed as Electrospun Nanofibers or Thermo-Compression Molded Film* *Materials Research*, vol. 19, no. 1, pp. 57-66, 2016.
[21] W. Penkhrue, D. Jendrossek, C. Khanongnuch, W. Pathom-Aree, T. Aizawa, R. L. Behrens and S. Lumyong, “Response surface method for polyhydroxybutyrate (PHB) bioplastic accumulation in Bacillus drentensis BP17 using pineapple peel”, *PloS one*, vol. 15, no. 3, pp. 0230443, 2020.

[22] M. Mahmoud, M. Berekaa and A. Al Thawadi, "Biosynthesis of polyhydroxybutyrate (PHB) biopolymer by Bacillus megaterium SW1-2: Application of Box-Behnkenesign for optimization of process parameters", *Microbiol Res*, vol. 6, no. 2, pp. 838-845, 2012.