

SYNTHESIS OF POLYHYDROXYBUTYRATE NANOPARTICLES USING SURFACTANT (SPAN20) FOR HYDROPHOBIC DRUG DELIVERY

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ABSTRACT

Biopolymers like polyhydroxybutyrate (PHB) are biocompatible and biodegradable, it forms nanoparticle when appropriate solvent systems are employed. Thus, they fulfill the major requirement as a drug carrier. In this study, surfactant (Span20) influenced PHB nanoparticles were prepared by nanoprecipitation method with different solvent systems i.e. Chloroform:DMSO (CD), Chloroform: Water (CW), Ethylacetate: DMSO (ED) and Ethylacetate:Water (EW). The nanoparticles were also loaded with a hydrophobic drug - curcumin. Further, the produced nanoparticles were characterized and utilized for in-vitro drug release studies. The nanoparticles were below 300nm in size and these nanoparticles were found to release the drug for longer duration i.e. more than 110 minutes. From the controlled release studies carried out against *Bacillus subtilis*, it was found that the nanoparticles released the encapsulated drug (Curcumin) efficiently, when acetic acid was used as the solvent system.

Keywords: Biopolymer, Nanoparticles, PHB, Surfactant.

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INTRODUCTION

Nanotechnology deals with the formation and utilization of particles from a broad range of sources and are used in a variety of fields, naming a few; agriculture¹, gene therapy², art conservation³, theranostics^{4,5} and drug delivery^{6,7}. Drug delivery and nanotechnology joined hands for novel reasons such as increased stability of the drug, higher surface area, decreased drug resistance and enhanced rate of dissolution, solubility, etc⁸. Majorly, the half-life of the poorly-water soluble drugs in systemic circulation is prolonged when it is encapsulated in nanoparticles⁹. It is also to be noted that, more than 20 nanoparticles are already in clinical use with respect to therapeutics, indicating the ability of these particles to enhance the value of drugs¹⁰. Though they prove to have such inestimable applications, they are still not accepted widely as they are toxic to some extent.¹¹⁻¹⁴ The nanoparticles acquire size depend on properties which also implies an effect on its toxic nature. A wide range of metal nanoparticles also shows some toxic effect to live cells¹⁵. To overcome this drawback, biopolymers are used as the base materials¹⁶. Biopolymers are known for their non-toxic nature, biodegradability and biocompatibility¹⁷. Polyhydroxyalkanoates (PHA) is one of the many biopolymers and is a linear chain of various ester groups that are produced in bacteria during physiologically stressful conditions as a carbon source and energy reserve. They exhibit good physiochemical properties that are exploited for many biomedical applications.¹⁸⁻²⁰ Polyhydroxybutyrate (PHB) is the first found, four carbon chained homopolymer under the category²¹ and it is universally known as an alternative for plastics¹⁸. PHB is being used as implantation material, biofuel etc.^{22,23} Nanoparticles were also prepared and researched widely from PHB with major application in drug delivery.²¹

Rasayan J. Chem., 11(4), 1686-1695(2018)

<http://dx.doi.org/10.31788/RJC.2018.1144053>



PHB nanoparticles loaded with rifampicin have been used in wound dressing with cotton gauze²⁴. These PHB based nanoparticles are commonly produced using various methods such as salting out, solvent evaporation, supercritical fluid technology, dialysis, nanoprecipitation, emulsion, etc²⁵. PHB nanoparticles are efficiently prepared using surfactants²⁶ and are also used for drug delivery²⁷. Utilizing surfactants like pluronic acid enhances the drug encapsulation and also has influence in size formation²⁸. In emulsion/solvent evaporation technique, PHA is dissolved in an organic solvent (preferably chloroform/DMSO) and then emulsified in water having surfactants; the solution is then evaporated to produce micro/nanoparticles²⁹. Parameters like the choice of solvent and surfactant concentration are crucial in size formation^{30,31} as this surfactant reduces the droplet coagulation and form stable nanometric particles³². Surfactants like SDS, Tween, pluronic acid and polyvinyl chloride are commonly used surfactants in the production of PHB based nanoparticles^{28,30}. Curcumin is a well-known drug which exhibits versatile bioactivities like antioxidant activity, anticancer activity, antibacterial activity, anti-inflammatory activity etc^{33,34}. But, these exemplary properties are blinded by its poor bioavailability and solubility that are due to factors such as administration route, elimination and rapid metabolism of curcumin in the system³⁵. Having known the importance of surfactants in the production of PHB nanoparticles especially in size reduction and drug encapsulation, we wanted to utilize Span 20 as surfactants rather using the common surfactants like Tween and SDS. In this study, various solvent systems were utilized, where PHB was dissolved in non-polar solvents (either chloroform or ethyl acetate) and this solution was dropped into anti-solvent (either water or DMSO) in presence of Span 20. Curcumin was loaded into PHB nanoparticles and the nanoparticles were characterized using FTIR, SEM and AFM. The efficiency of the nanoparticle to encapsulate and release the loaded drug was also studied.

EXPERIMENTAL

Materials Required

Polyhydroxybutyrate (PHB) (derived from microbial fermentation) (Sigma-Aldrich, India), Chloroform (Qualigens, India), Ethylacetate (Qualigens, India), DMSO (Qualigens, India), Curcumin (SRL, India), Span20 (HiMedia, India). Distilled water was used all through the experiment. All the chemicals were either analytical grade or extra pure.

Synthesis of PHB Nanoparticles

PHB nanoparticles were synthesized by a slight modification of the nanoprecipitation method explained by Shakeri et al³⁶ wherein, 0.05% PHB was dissolved in the respective solvent (chloroform/ethyl acetate) and heated. Later the above mixture was suspended into the anti-solvent (water/DMSO) under magnetic stirring, subsequently 0.1% Span 20 was added drop-wise. The precipitates so formed were collected and lyophilized for further usage. To prepare curcumin loaded nanoparticles, 2.5mg of curcumin was initially added to the non-polar solvents, then it was performed to form nanoparticle as described earlier.

Characterization of PHB Nanoparticles

Fourier Transform Infrared Spectroscopy (FTIR)

Various functional groups present in the prepared PHB nanoparticle was identified using the different modes of vibrations³⁷. 1 to 2% of PHB nanoparticles was added to KBr solution was heated and ground to obtain a homogenous mixture. This mixture was plated into KBr discs using standard methods³⁸. They were then encountered with a spectrum ranging from 4000 to 500 cm⁻¹ IR Affinity-1s (Shimadzu, Japan) in transmission mode. A graph was plotted against wavelength and transmission rate.

Scanning Electron Microscopy (SEM)

In order to measure the size and visualize the distribution of the particles, the PHB nanoparticles were diluted to a concentration of 1mg/ml by dispersing them into deionized water and a drop of it was mounted on a glass slide which was embedded on stubs and dried at room temperature. Later they were sputtered with gold and viewed under SEM (SEI and BSI).

Atomic Force Microscopy

A thin layer of nanoparticle was mounted onto a glass slide and viewed in atomic force microscopy (Bruker, Germany) by which the surface topography of the nanoparticles was visualized.

Drug Encapsulation Efficiency

Immediately after producing the curcumin loaded nanoparticles, 1ml of the supernatant was aspirated after centrifugation at 5000 rpm. This was performed for every 10 minutes and checked for absorbance at 421nm using UV visible spectrophotometer (Systronics), as 421nm is the absorbance maxima of curcumin³⁹. A graph was plotted against time and absorbance to identify the efficiency of the nanoparticles to encapsulate the drug with respect to time.

In Vitro Drug Release Kinetics

The ability of the nanoparticle to release the encapsulated drug was checked using the dialysis method⁴⁰. Dialysis membrane containing a suspension of nanoparticle in PBS (pH 7) in the ratio 0.5:1 was placed in acidic PBS solution (pH 3) at room temperature and every 10 min, 1 ml of the external solution was collected up to 180 min. They were then checked for the absorbance of curcumin by measuring the absorbance using UV visible spectrophotometer (Systronics) at a wavelength of 421nm³⁹. A graph against absorbance and time was plotted.

In-Vitro Controlled Release Studies

Controlled release study was performed using agar well diffusion method. In this study, two different solvents- PBS (pH 7) or acetic acid (1%) were utilized to dissolve the nanoparticles. Nutrient agar plates were swabbed all over with *Bacillus subtilis* and wells were bored. Different concentrations (2, 4, 6, 8µg/ml) of nanoparticles dispersed in respective solvents were added to the wells⁴¹. Erythromycin was used as positive control and the respective solvent as a negative control. The plates were incubated for 24h and the zone of inhibition was recorded.

RESULTS AND DISCUSSION

Fourier Transform Infrared Spectroscopy (FTIR)

Strong peaks at 1724-1760 cm⁻¹ shows the presence of C=O which is the characteristic of PHB and peaks at 2983-2852 cm⁻¹ shows the presence of alkyl C-H bond (Fig.-1)^{41,42}. The presence of curcumin in loaded samples (Fig.-1b, d, f, h) was confirmed by the presence of peaks at 3503-3436 cm⁻¹ for the presence of OH bond whereas, 980-678 cm⁻¹ was indicating the =C-H bend. 1594-1432 cm⁻¹ was showing the C-C bond and 1051 to 1229 cm⁻¹ was showing the presence of C-O bond⁴³. C-O-H bond of curcumin was found at 1375 cm⁻¹ to 1351 cm⁻¹. C-O of curcumin was found between 1194 cm⁻¹ and 1184 cm⁻¹ (Fig.-1b, d, f, h)⁴⁴.

Scanning Electron Microscopy (SEM)

All the nanoparticles prepared in this study were spherical in shape. The unloaded nanoparticles prepared using Chloroform:DMSO was in size ranging from 104 nm to 400 nm, where the loaded were between 200 and 600nm (Fig.-2a, b), the unloaded particles of Chloroform:water was ranging from 48 nm to 75 nm, while it was loaded with drug, it increased the size to 350 - 700nm (Fig.-2c, d). The drug loaded and unloaded particles prepared using Ethyl Acetate:DMSO had size around 157nm to 550nm (Fig.-2e, f) and the size of Ethyl Acetate:Water was 98 nm to 361 nm (Fig.-2g, h). The shape was uniformly spherical in the nanoparticles prepared using DMSO as anti-solvent. Drug-loaded nanoparticles formed using the water and ethyl acetate was found to be aggregated and non-uniform in shape. Span was found to produce a particle of smaller size than the other surfactant utilized preparations. In our earlier report, we have produced polyhydroxyalkanoate nanoparticles without any surfactant and the minimal size we could achieve was around 290nm⁴⁵. Thus it is proving that Span 20 was having influence in size reduction, even in most cases it was producing spherical shaped nanoparticles. There are reports saying that surfactant influences nanoparticles³³, where 250-710 nm and below 300nm sized PHB nanoparticle were produced using SDS⁴⁵ and PVA as surfactant⁴⁵, where Sasikumar et al⁴⁷ have achieved to produce 100nm sized PHB nanoparticles with TWEEN 80 by nanoprecipitation method. Increasing concentration of surfactant (TWEEN 80) was found to decrease the size of PHB nanoparticles³⁶. Drug-loaded nanoparticle was looking bigger than the unloaded one. Increasing the concentration of organic phase has been reported to influence in the reduction of the size of PHB based nanoparticles⁴⁸.

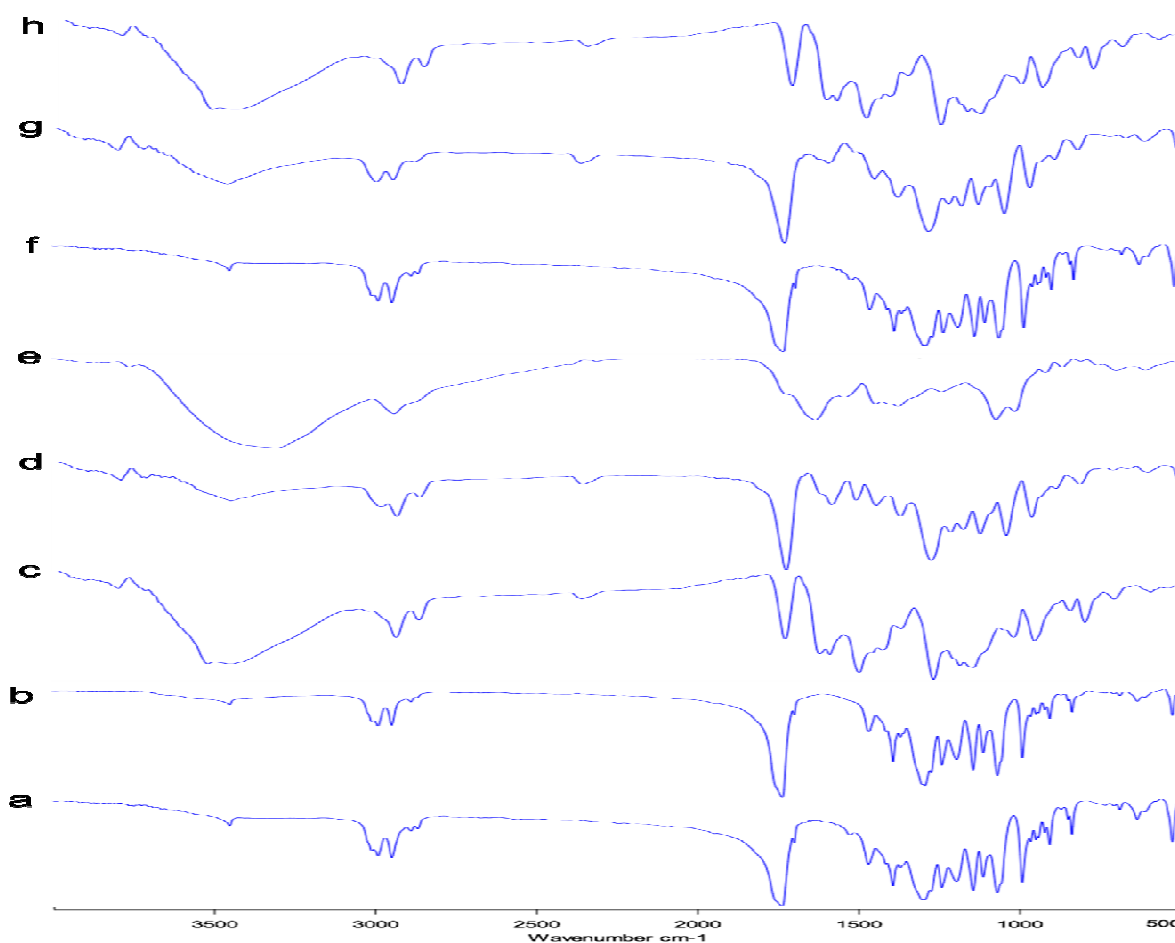


Fig.-1: FTIR Analysis: Nanoparticles prepared using (a) Chloroform and DMSO, (b) Curcumin, Chloroform and DMSO, (c) Chloroform and Water, (d) Curcumin, Chloroform and water, (e) Ethyl acetate and DMSO, (f) Curcumin, Ethyl acetate and DMSO, (g) Ethyl acetate and Water, (h) Curcumin, Ethyl acetate and Water

Atomic Force Microscopy (AFM)

From AFM analysis, all the samples were found to form smooth, spherical shaped and aggregated nanoparticles. Smallest particles of size ranging between 48 and 68nm was produced while Chloroform-DMSO was used (Fig.-3a, b); 41nm and 96nm for nanoparticles prepared using Chloroform-Water (Fig.-3c, d); 24nm and 53nm for Ethyl Acetate-DMSO as solvent system (Fig.-3e, f) and 60 to 96nm for Ethyl Acetate – Water as solvent system (Fig.-3g, h). Surfactants like tween and SDS were forming bigger sized micro/nanoparticles^{36,45-47}. Size of the particles was reported as decreasing while the surfactant concentration was increasing³⁶. Kilicay et al⁴⁹ found PHB based nanoparticles produced by transesterification process to be a size between 230 and 235nm by AFM analysis.

Drug Encapsulation Efficiency

After characterization, the nanoparticles were utilized for curcumin encapsulation. The nanoparticles were found to encapsulate the curcumin in a constant fashion except for the nanoparticles formed using chloroform-DMSO (Fig.-4). Encapsulation was found to be increasing as time duration increased. Nanoparticles prepared using Chloroform – Water, encapsulated drug efficiently and better. Surfactants were reported to enhance the drug loading efficiency^{30,31}. In a study, it was found that the encapsulation of curcumin into PHA nanoparticles was slower when surfactant was not used⁴⁴. Shakeriet al³⁶ have loaded lipophilic carvacrol into PHB based nanoparticles and the loading efficiency was found to be 21%. Even

in our earlier study, we found polyhydroxyalkanoate based nanoparticles to encapsulate curcumin efficiently⁴⁴.

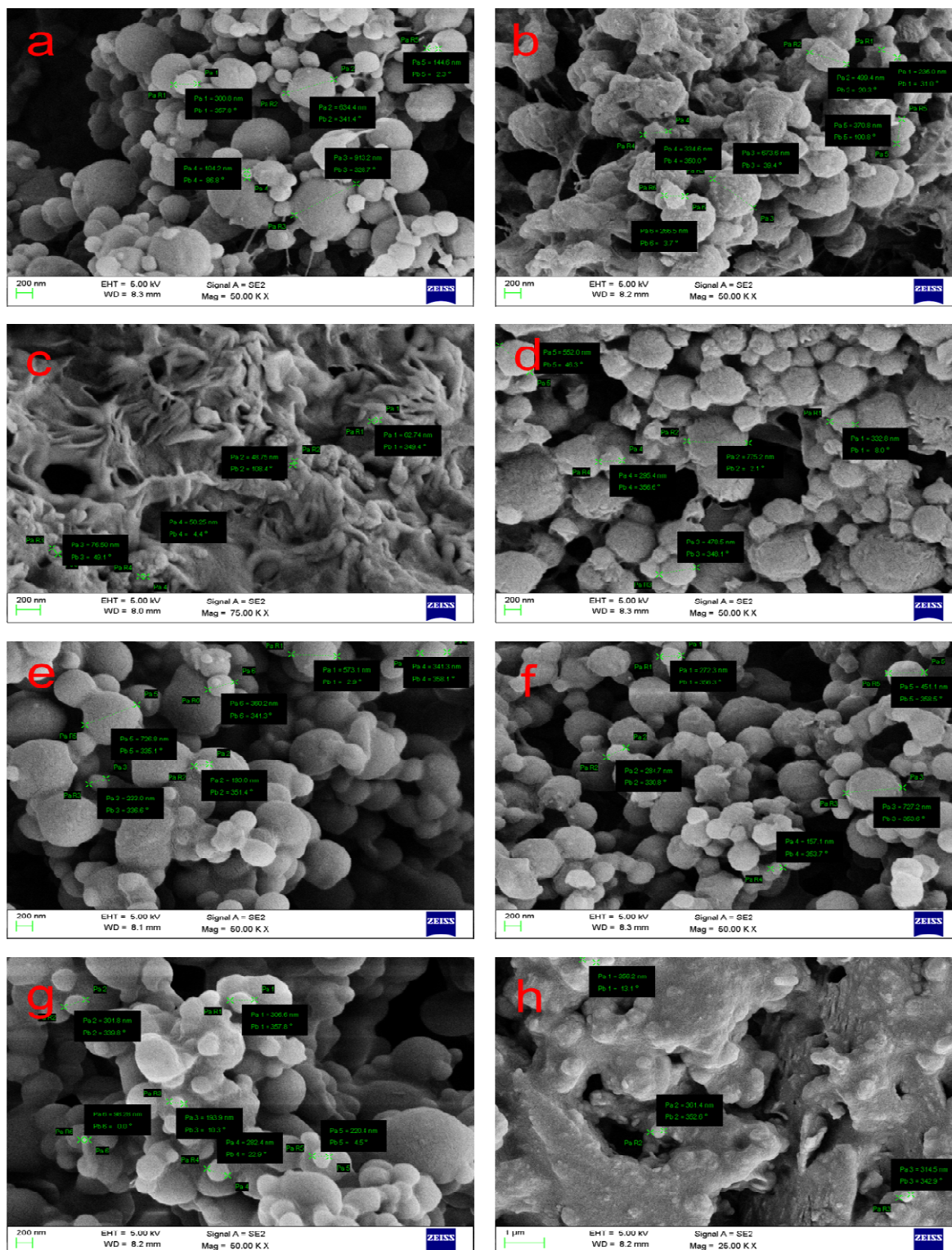


Fig.-2: SEM Analysis: Nanoparticles prepared using (a) Chloroform and DMSO, (b) Loaded, Chloroform and DMSO, (c) Chloroform and Water, (d) Loaded, Chloroform and Water, (e) Ethyl acetate and DMSO, (f) Loaded, Ethyl acetate and DMSO, (g) Ethyl acetate and Water, (h) Loaded, Ethyl acetate and Water

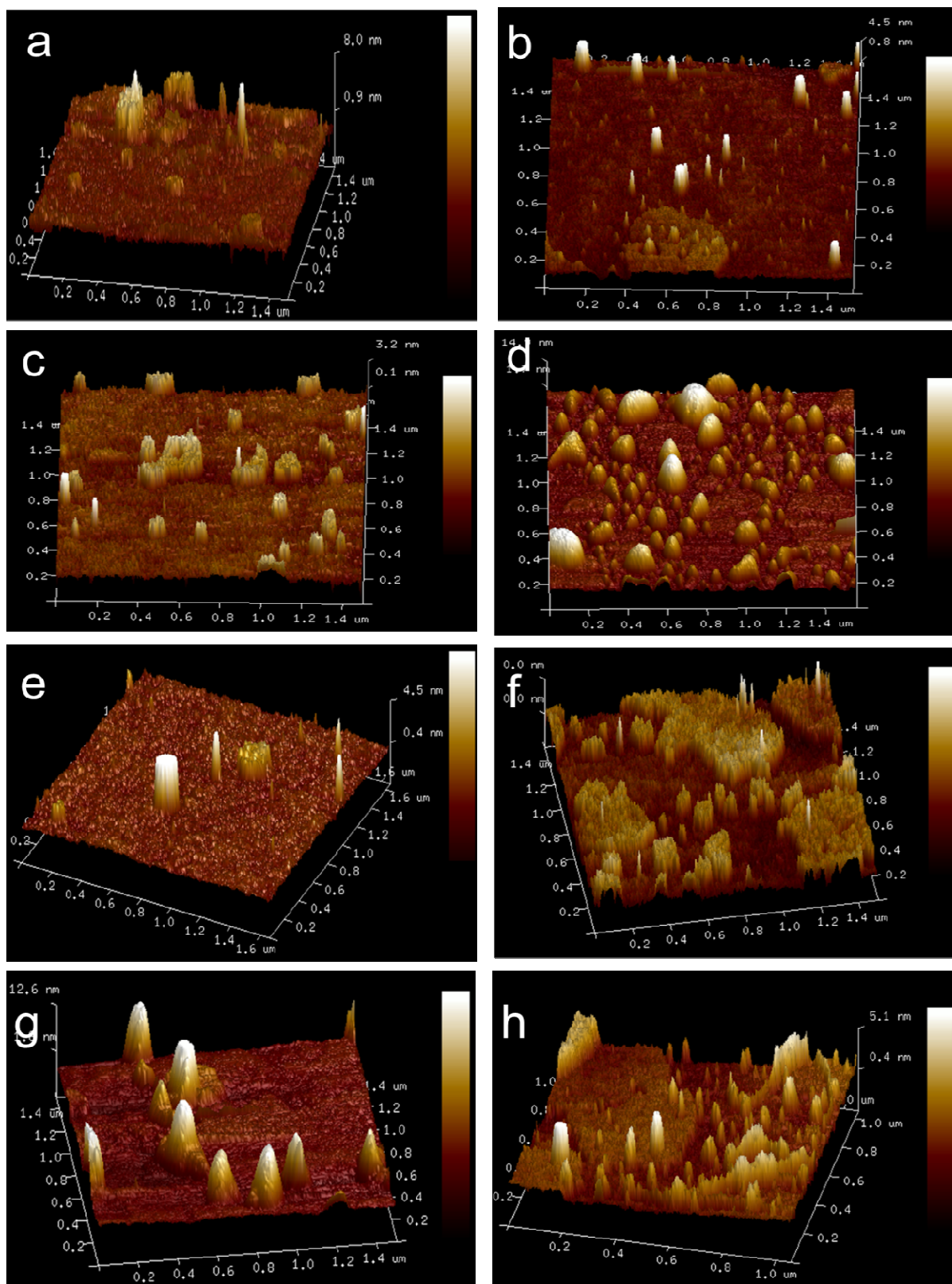


Fig.-3: AFM analysis Nanoparticles prepared using (a) Chloroform and DMSO, (b) Loaded, Chloroform and DMSO, (c) Chloroform and Water, (d) Loaded, Chloroform and water, (e) Ethyl acetate and DMSO, (f) Loaded, Ethyl acetate and DMSO, (g) Ethyl acetate and Water, (h) Loaded, Ethyl acetate and Water

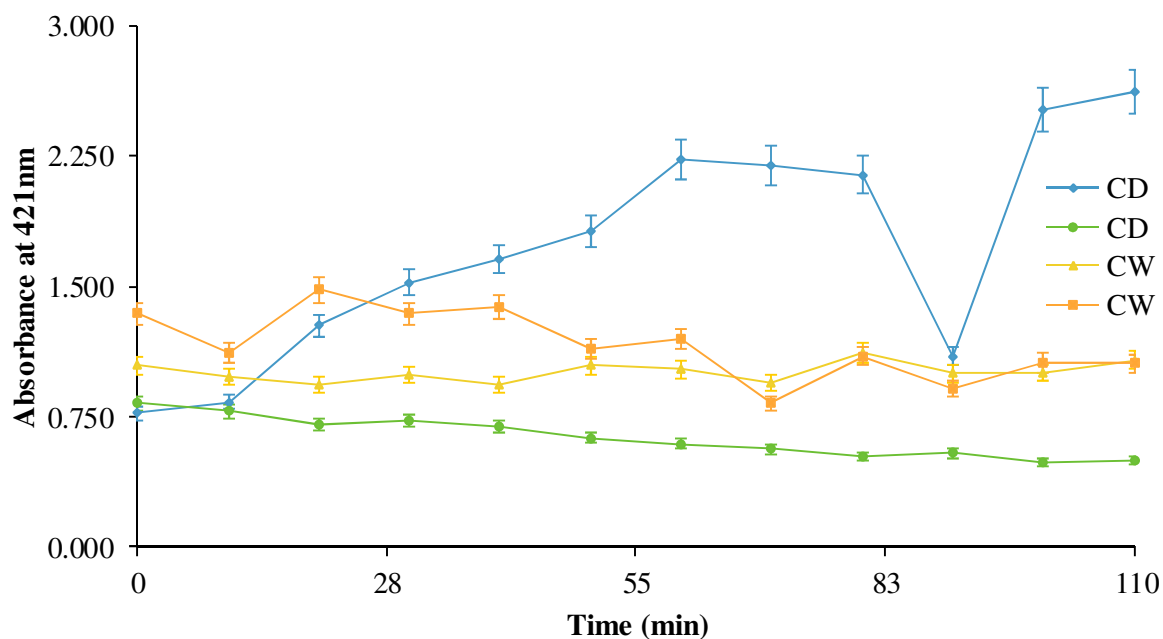


Fig.-4: Drug Encapsulation Efficiency Studies.

CD- Chloroform:DMSO, CW – Chloroform:Water, ED – Ethylacetate:DMSO, EW - Ethylacetate:Water

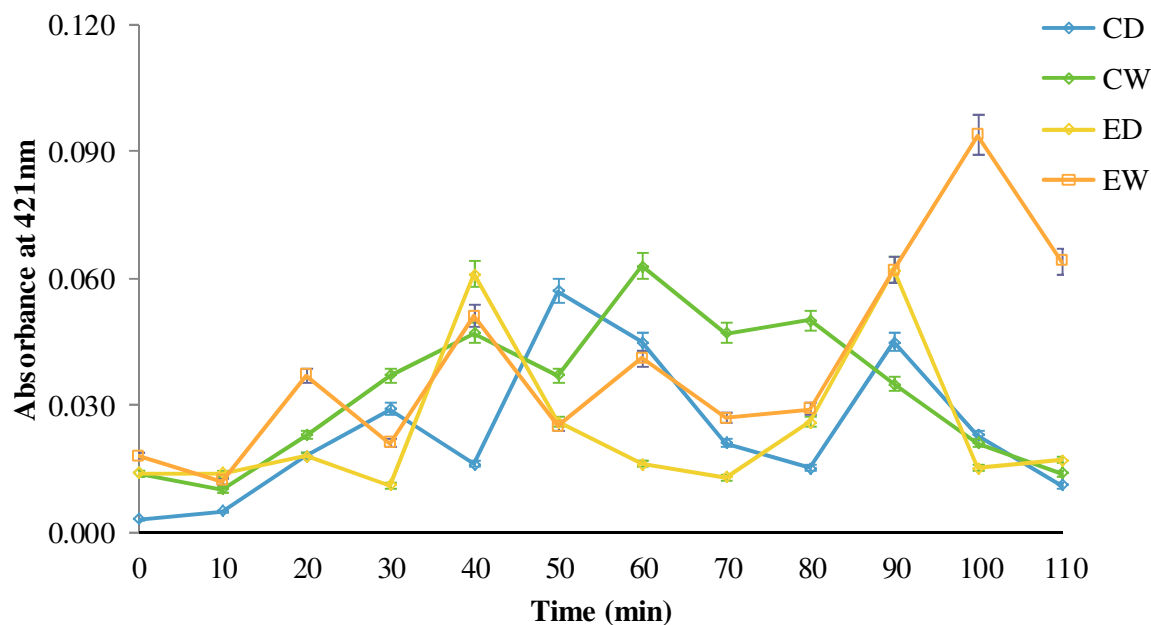


Fig.-5: In Vitro Drug Release Kinetics of PHB Nanoparticles, CD- Chloroform:DMSO, CW – Chloroform:Water, ED – Ethylacetate:DMSO, EW - Ethylacetate:Water

In Vitro Drug Release Kinetics

Nanoparticles produced using ethyl acetate: DMSO was found to release the drug faster i.e. in the 70th min, where the other particles were releasing for longer time i.e. more than 110 min (Fig.-5). The drug-loaded PHB nanoparticle was tended to release drug at pH 3; even in our previous study⁴⁴ we found the polyhydroxyalkanoate based nanoparticles to release curcumin at pH 3 only. These nanoparticles tend to react with the acidic environment and disperse the loaded drug. Nachiyar et al⁴⁶ reported that PHB

nanoparticles produced using PVA as a surfactant to release levofloxacin for 24h. Sasikumar et al⁴⁷ found PHB nanoparticles produced using TWEEN 80 as a surfactant to release doxorubicin for more than 72h.

Table-1: Anti-Bacterial Activity of PHB Nanoparticles against *Bacillus subtilis*

Solvent System	+ve Control	+ve Control	-ve Control	2 µg	4 µg	6 µg	8 µg
PBS	Chloroform-DMSO	2.6	-	-	-	-	-
	Chloroform-Water	2.6	-	-	-	-	-
	Ethyl acetate-DMSO	2.6	-	-	-	-	-
	Ethyl acetate-water	2.6	-	-	-	-	-
Acetic acid	Chloroform-DMSO	2.6	-	-	-	0.5	0.7
	Chloroform-Water	2.6	-	-	-	-	0.7
	Ethyl acetate-DMSO	2.6	-	-	-	0.5	0.7
	Ethyl acetate-water	2.6	-	-	-	0.5	0.7

In-Vitro Controlled release Studies

When the nanoparticles were dissolved in PBS, they did not show any zone which is in accordance with the earlier reports. When acetic acid was used, there was a slight increase in the activity (Table-1). The increase in the activity might be due to the breakdown of the outer PHB by the non-polar nature of acetic acid which would have caused the release of the encapsulated drug⁴². The effect could have also been due to the change in pH which is seen evidently from the drug release kinetics data (Fig.-5).

CONCLUSION

In this study, commercially available PHB was utilized for nanoparticle synthesis with different solvent system i.e. chloroform:DMSO, chloroform:water, ethyl acetate:DMSO and ethyl acetate:water and span20 as a surfactant and also utilized for loading hydrophobic drug – curcumin. Spherically shaped nanoparticles of size ranging from 40 to 100nm were produced using the surfactant Span 20, which was confirmed by SEM and AFM analysis. Span 20 has influence in the drug encapsulation. Further, it was utilized for drug release studies, where it was released for more than 110 minutes. When acetic acid was used as a solvent, the nanoparticles released the drug efficiently which was confirmed by drug release kinetics and in-vitro controlled release study showing that acetic acid has an effect on the outer PHB nanoparticle which has led to the release of the encapsulated drug.

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