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Characterization of anti-cancer drug materials loaded poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) microspheres for drug delivery system in biochemical material system

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Abstract. Poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) is one of the components of polyhydroxyalkanoates (PHAs) and some of its mechanical properties have been shown to improve over poly (3-hydroxybutyrate) (PHB) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). The investigation of PHBHHx microspheres as a drug delivery system was prepared by emulsion-solvent evaporation method for the sustained release of anti-cancer drug 5-fluorouracil (5-FU) and cyclosporin A (CsA). The mean diameter of the PHBHHx microspheres ranged from 5.24 to 22.10 μm dependent on the different processing parameters. The PHBHHx concentration, emulsifier concentration, anti-cancer drug dosage, and agitation speed, were optimized according to the encapsulation efficiency of 4% PHBHHx, 0.5% SDS, 10 mg anti-cancer drug, and 500 rpm. Under optimized conditions, the encapsulation efficiency of 5-FU and CsA microspheres were 7.19% and 96.44%, respectively. The morphologies of scanning electron microscope (SEM) suggested that PHBHHx microspheres were relatively smooth that provided better dispersion compared to PHB microspheres. The in vitro release profiles indicated 32.42% of 5-FU and 30.61% of CsA were released from PHBHHx microspheres during the initial burst phase, and the drug release from PHBHHx microsphere could be detected even after one month. The characteristics of PHBHHx microspheres demonstrated the feasibility of PHBHHx microsphere as a novel matrix for drug release system. With positive maintenance of the therapeutic concentrations of the drug, side effects can be reduced and patient compliance can be improved.

Introduction

Microspheres between 1 and 1000 μm, have been widely studied in the pharmaceutical field for drug release due to the advantages over conventional pharmaceutical formulations[1]. These advantages include lower variability between patient responses[2], lower risk of dose dumping[3], and higher patient comfort and compliance[4]. In general, the microsphere matrices are composed of biodegradable and biocompatible polymers, such as poly (glycolide) (PGA), poly (lactic acid) (PLA), poly (lactide-co-glycolide) (PLGA), poly (ε-caprolactone) (PCL), poly (3-hydroxybutyrate) (PHB), poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), gelatin, chitosan (CHI), alginate (ALG) and so on[5-7]. At the present time, biocompatible and biodegradable matrices have been successfully used to prepare pain killers, antidepressants, contraceptives, anti-cancer, and anti-inflammatory drugs[8].

The first and most often reported polyhydroxyalkanoates (PHA) is poly (3-hydroxybutyrate) (PHB). The degradation of PHB leads to the formation of 3-hydroxybutiric acid, a normal constituent of blood[9], which suggests that PHB will be well tolerated in vivo. Microspheres prepared from PHB have been investigated as matrix for several drugs. However, its high degree of crystallinity, ranging from 60 to 90%, leads to the formation of porous microspheres. Therefore, PHB results in high initial

drug release (burst effect) incapable of sustaining long period release. It has been assumed that the release phenomenon is more dependent on drug dissolution rather than matrix degradation and diffusion. Poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) is composed of both short-chain-length monomer 3-hydroxybutyrate (3HB) and medium-chain-length monomer 3-hydroxyhexanoate (3HHx)[10]. PHBHHx was reported to possess similar mechanical properties to allow low density polyethylene and the copolymer films show a high degree of elongation tolerance, an improvement PHB[11].

In this study, $PHBHH_X$ matrix in microspheres was prepared with 5-fluorouracil (5-FU) and cyclosporin A (CsA) for a sustained release system for comparison against PHB. It is possible to allow the maintenance of the therapeutic concentrations of the drug, reduce the side effects, and improve patient compliance.

Materials and Methods

Reagents and Chemicals

PHB was purchased from Sigma Chem. Co. (USA). 5-fluorouracil (5-FU) and cyclosporin A (CsA) were purchased from Sayon Biotech Co. Ltd. (China). All other chemicals and solvents were reagent grade.

Production of PHBHHx

Aeromonas hydrophila WQ strain was cultivated in mineral medium. 10 g/L Lauric acid for *A. hydrophila* cultivation was served as carbon source. The cultures were incubated in 30 L fermentor containing 15 L culture broth under 30°C. Cells were harvested by centrifugation after 72 h and lyophilized. Gas chromatographic (GC) analysis of intracellular PHA content and PHA composition was performed as described previously[12].

Preparation of microspheres

Different formulations of 5-FU loaded microspheres were prepared by an oil-in water (O/W) emulsion-solvent evaporation method[13]. Briefly, a certain amount of 5-FU, 20 mg of phospholipid, and 10 mg of cholesterol were dissolved in an aqueous solution of 30 ml tetrahydrofuran, then they were evaporated to dryness using Rotary Evaporators. 200 mg PHBHHx powders were dissolved in dichloromethane with above mentioned compounds as oil phase, and the resulting solution was emulsified in an aqueous phase containing SDS as external water phase which was maintained under magnetic stirring at room temperature until the evaporation of the organic solvent. The resulting suspensions were centrifuged at 12000 rpm for 10 min, washed three times with distilled water. After that, microspheres were freeze-dried and stored under vacuum for further use. The PHB microspheres were prepared using the same method.

Similarly, 200 mg PHBHHx powders were dissolved in dichloromethane with CsA as oil phase[14], which was emulsified in 50 ml of an aqueous phase containing SDS, and the following steps were the same as above.

Determination of the encapsulation efficiency

To determine 5-FU and CsA encapsulation efficiency in the microsphere formulations, 10 mg of microspheres were dissolved in 1 ml of chloroform, and then 9 ml of ethanol were added to precipitate the polymer. Subsequently, the sample was centrifuged at 12000 rpm for 10 min to spin down the precipitated polymer[15]. The 5-FU and CsA concentration in the supernatant was determined by UV spectroscopy at 265 nm and 232 nm, respectively. The drugs content were quantified against a standard curve prepared by dissolving drugs in the same solvent. The encapsulation efficiency (%) was calculated as the difference between the amount of drug initially added to the formulation and the amount found in the microspheres after the UV analysis.

Characterization of the microspheres by SEM

The morphologies and structures of microspheres [16]were characterized using a KYKY-EM3200 digitization scanning electron microscope (KYKY Technology Development LTD, Beijing, China).

Analyses of microspheres size

Freeze-dried microspheres were re-dispersed in distilled water and observed by microscopy (Olympus, Japan) to determine the mean particle size[17].

In vitro release

Drug release assays were carried out using the dialyses method[18]. 20 mg of 5-FU loaded microspheres were placed in a dialyses bag with 3 ml of pH 7.4 phosphate buffer. The sample filled tubes were hermetically sealed and immersed into 50 ml of pH 7.4 phosphate buffer maintained at 37°C under magnetic stirring at 50 rpm in a water bath chader. Aliquots of the medium were withdrawn at regular time intervals and analyzed for 5-FU concentration by UV spectrophotometry at 265 nm.

Similarly, 20 mg of CsA-loaded microspheres were placed in a dialyses bag with 1 ml of pH 7.4 phosphate buffer mixed with 25% ethanol[14]. The sample filled tubes were hermetically sealed and immersed into 20 ml of pH 7.4 phosphate buffer mix with 25% ethanol maintained at 37°C under magnetic stirring at 50 rpm in a water bath chader. Aliquots of the medium were withdrawn at regular time intervals and analyzed for CsA concentration by UV spectrophotometry at 203 nm.

Results and discussion

Preparation for PHBHHx microspheres

The PHBHHx microspheres were prepared by emulsion-solvent evaporation. During the process, several parameters such as polymer concentration in the oil phase, concentration of the emulsifier in the external water phase, drug dosage, and agitation speed were changed. The results are summarized in Table 1.

As expected, the average particle size and encapsulation efficiency of microspheres increased when the agitation speed changed from 1000 to 500 rpm. Similar results by Khang[15]for PHBV encapsulation of 5-FU were obtained. High agitation speed made emulsion droplets smaller and more uniform, which led to smaller average particle size and span of microspheres.

Generally, the viscosity of a polymer solution has a significant effect on the sizes of the resultant microspheres. When the PHBHHx concentration increased from 2.0% to 4.0%, the average sizes of PHBHHx microspheres increased from 8.18 to 12.73 μm due to low concentration of PHBHHx in the system, repressed by the aggregation of droplets. As a consequence, the large amount of the polymer increases the viscosity of the drops and consequently decreases the speed of mass transfer contributing to a high drug encapsulation. A similar behavior was found using the polymers PHB and PHBV[16].

While maintaining the same PHBHHx and emulsifier concentrations (4.0% and 1.0%, respectively) and decreasing the initial feeding amount of 5-FU (30.0 mg to 10.0 mg), the encapsulation efficiency increased from 4.41% to 8.52%. This observation can be explained by the constant polymer amount used in all of the formulations and the increased drug loss from the polymer matrix to the outer aqueous phase from the enlarged concentration gradient resulted with the increased drug amounts in microspheres in the case of higher initial feeding amount of drug in the fabrication process. Similar results by Lu[19] and Kılıçay[20] were obtained. At the same time, the mean diameter was increased from 12.73 to 22.10 μm.

In the solvent-evaporation method, the emulsifier is used to stabilize the suspended droplets over the whole process[21]. In the present work, decreasing SDS concentration from 1.0 to 0.5% decreased the encapsulation efficiency from 8.52 to 7.19% and the mean size from 22.10 to 20.52 μm. Generally, a higher emulsifier concentration could increase viscosity of the external water phase and prevent emulsion droplets from coalescence, resulting in smaller emulsion droplets. However,

microsphere size increased in present work, which is contributed by 0.5% SDS achieving the purpose of emulsification. With the concentration increasing, the excess SDS assembled to be a large number of SDS micelles, leading to the formation of larger microspheres, and average diameter of microspheres increased. These results showed that suitable concentration of emulsifier should be used in the preparation of microspheres to obtain desired size and release.

Table 1 Processing parameters and properties of PHBHHX microspheres

Formulation ID	5-FU [mg]	PHBHHx [%]	SDS [%]	Agitation speed [rpm]	EE [%]	Mean diameter [μm]	Span
A	30	2.0	1.0	1000	1.18%	5.24	1.41
B	30	2.0	1.0	500	3.04%	8.18	1.47
C	30	4.0	1.0	500	4.41%	12.73	1.84
D	20	4.0	1.0	500	4.86%	18.97	1.85
E	10	4.0	1.0	500	8.52%	22.10	1.73
F	10	4.0	0.5	500	7.19%	20.52	1.32

Effect of drug and matrix material to microspheres

According to processing parameters research, we received the optimal preparation conditions，as 4% PHBHHx, 10mg anti-cancer drug, 0.5% SDS and 500 rpm. Under these conditions, we compared the characteristics of PHBHHx and PHB as the matrix material in microspheres prepared with 5-fluorouracil (5-FU) and cyclosporin A (CsA) for sustained release systems. The results are summarized in Table 2.

The encapsulation efficiency of 5-FU PHBHHx microspheres is very low, which is similar as Li's findings[22]. One of the reasons for low encapsulation efficiency is because 5-FU as a crystalline powder, not suitable for the drug delivery due to massive drugs have not realized encapsulation, the other is that 5-FU is hydrophilic and penetrate into the external phase during solvent evaporation and washing process. High CsA encapsulation efficiency has been achieved (96.44%) like many other reports[23]in this study. CsA solubility in water is very low, only 4 μg/ml[24], owing to its high hydrophobic nature. But lipophilic CsA is more prone to distribute in the loading matrix of hydrophobic polymers than in external aqueous. Our results showed that lipophilic drug was very suitable for loaded into microspheres by emulsion-solvent evaporation method.

Table 2 Effect of PHBHHx and PHB on the properties of microspheres

Drug	Polymer	EE [%]	Mean diameter [μm]	Span
5-FU	$PHBHH_X$	7.19	20.52	1.32
	PHB	6.80	17.29	1.27
CsA	$PHBHH_X$	96.44	18.29	1.48
	PHB	94.13	20.37	1.64

Surface Characterization Studies

The representative SEM images of microspheres are shown in Fig.1. SEM analyses revealed the detailed surface morphologies of the PHBHHx and PHB microspheres. The photographs indicated that both of the PHB and PHBHHx microspheres exhibited a spherical shape. The surface of PHBHHx microspheres was relatively smooth, and few pores were observed [Fig. (C1), (D1)]. In addition, microspheres had relatively uniform size and dispersion, and agglomeration was not observed [Fig. (C2), (D2)]. The surface of PHB microspheres was rougher with increased micro-size pores [Fig. (A1), (B1)]. Microspheres had relatively uniform size, but had poor dispersion under large number of microspheres sticking together [Fig. (A2), (B2)]. The distinct differences can be attributed

to the different physicochemical characters between PHB and PHBHHx. The result of PHB microsphere consists with the findings of Bazzo[25], and Bidone[26]. After solvent removal from the internal phase of the emulsion, the roughness of the particle surface of the resulting PHB microspheres has been associated to the nature of the material, high crystallinity (60 to 90%), and fast precipitation. The crystallinity degree decreased when the 3HHx content increased in the copolymer. The low degree of crystallization produces less stress during the solvent evaporation process and the slow crystallization process provided the polymer with enough time to rearrange the molecules to release the stress produced during crystallization[27], which also had some effects in vitro release.

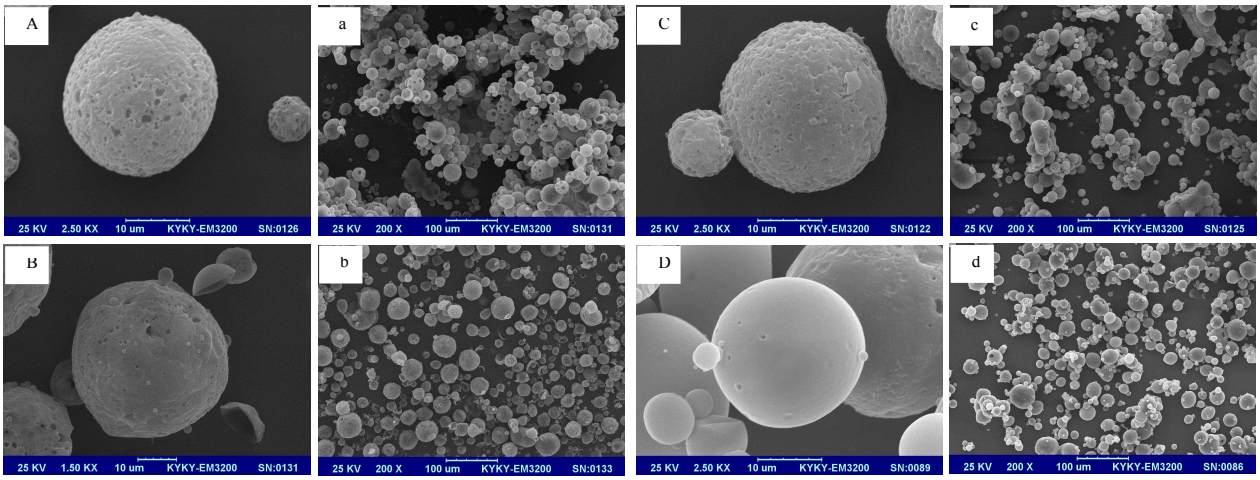

Fig.1 SEM micrographs of microspheres. (A) and (a) 5-Fu-Loaded PHB microspheres; (B) and (b) 5-Fu-Loaded PHBHH$_X$ microspheres; (C) and (c) CsA-Loaded PHB microspheres; (D) and (d) CsA-Loaded PHBHH$_X$ microspheres.

In vitro release studies

PHBHHx and PHB microsphere drug release profiles are displayed in Fig.2. Overall, cumulative release of 5-FU or CsA from PHBHHx and PHB microspheres demonstrated similar biphasic release profiles with an initial burst phase followed by a phase of slow sustained release. The drug loading in PHB microsphere produced a larger burst phase of release, leading to higher overall drug released. After a fast release during the first 24 h of assay, 54.28% of CsA and 49.76% of 5-FU were released from PHB microspheres. Similar results by Wang[28]for PHB encapsulation of levonorgestrol were obtained. When microspheres were prepared from PHBHHx, a significant change in the amount of released could be observed. Around 30.61% of CsA and 32.42% of 5-FU were released. It is generally accepted that drug release rate from microspheres is strongly dependent on polymer crystalline behavior and drug dispersion state the crystallization of the polymers during microsphere formation may produce micro voids in the microspheres, which can function as channels for water penetration [29]. Usually, an initial burst release is due to a rapid dissolution of drugs at or close to the particle surface and a subsequent exponential release due to drug diffusion from the interior of the particle[15, 30-31]. PHB, as a biodegradable polymer, is generally degraded very slowly in vitro[32]. The rate of drug diffusion was substantially higher than that of polymer degradation, so the release profiles are more dependent on drug diffusion rather than on polymer degradation[17]. The faster drug release of PHB microspheres may be caused by the porosity of the microparticle. Therefore the facilitation of drug release is dependent on the proximity of drug molecules near to the matrix surface and high porosity.

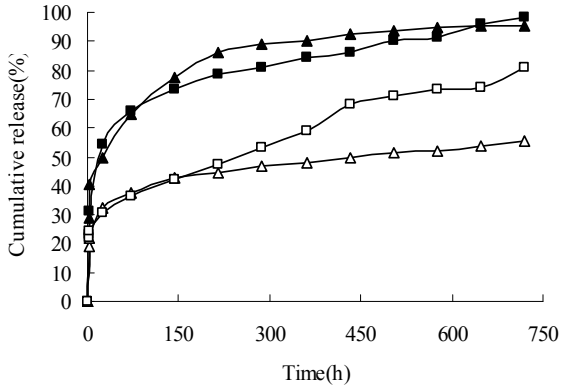

Fig.2 Cumulative in vitro release profiles of PHBHHx and PHB microspheres in pH 7.4 media at 37°C: ▲, 5-FU loaded PHB microspheres; △, 5-FU loaded PHBHHx microspheres; ■, CsA loaded PHB microspheres; □, CsA loaded PHBHHx microspheres;

Conclusions

In this study, the anti-cancer drug 5-FU and CsA were successfully loaded into PHBHHx microspheres, producing significant drug loading and encapsulation efficiency compared with PHB, using the emulsion-solvent evaporation technique. The morphology and in vitro release patterns were investigated to show that PHBHHx microspheres were spherical in shape, had narrow size distribution and relatively small size range. In conclusion, this study demonstrates the potential of PHBHHx as an effective biodegradable polymeric matrix for a promising sustained degradable device to reduce the strong side effects and to improve patient compliance. These characterized properties of PHBHHx microspheres may allow more diverse application in biomedical fields.

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