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Development of tunable biodegradable polyhydroxyalkanoates microspheres for controlled delivery of tetracycline for treating periodontal disease

Nootchanartch Panith,[1] Apinya Assavanig,[1] Sittiwat Lertsiri,[1] Magnus Bergkvist,[2] Rudee Surarit,[3] Nuttawee Niamsiri[1]

[1]Department of Biotechnology Faculty of Science, Mahidol University, Bangkok 10400, Thailand
[2]Colleges of Nanoscale Science and Engineering, SUNY Polytechnic Institute, Albany New York 12203
[3]Department of Oral Biology Faculty of Dentistry, Mahidol University, Bangkok 10400, Thailand
Correspondence to: N. Niamsiri (E-mail: nuttawee.nia@mahidol.ac.th)

ABSTRACT: This article was aimed at preparation and characterization of drug delivery carriers made from biodegradable polyhydroxyalkanoates (PHAs) for slow release of tetracycline (TC) for periodontal treatment. Four PHA variants; polyhydroxybutyrate (PHB), poly(hydroxybutyrate-co-hydroxyvalerate) with 5, 12, and 50% hydroxyvalerate were used to formulate TC-loaded PHA microspheres by double emulsion-solvent evaporation method. We also compared the effect of different molecular weight (M_w) of polyvinyl alcohol (PVA) acting as surface stabilizer on particle size, drug loading, encapsulation efficiency, and drug release profile. The TC-loaded PHA microspheres exhibited microscale and nanoscale spherical morphology under scanning electron microscopy. Among formulations, TC-loaded PHB:low M_w PVA demonstrated the highest TC loading with slow release behavior. Our results showed that the release rate from PHA microspheres was influenced by both the type of PHA and M_w of PVA stabilizer. Lastly, TC-loaded PHB microspheres showed efficient killing activity against periodontitis-causing bacteria, suggesting its potential application for treating periodontal disease. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 44128.

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INTRODUCTION

Periodontitis is a disease that affects the gum and other tissues supporting teeth.[1] It is caused by bacterial infection, which can initiate the destruction of both gingival and periodontal tissues and ultimately lead to tooth loss. Treatments of periodontal disease aim to reduce the infection by eliminating periodontitis-causing bacteria using a combination of mechanical cleaning and systemic antibiotic drug administration.[2,3] Tetracycline (TC) is a broad spectrum antibiotic that has been used extensively in the treatment of periodontal disease, since it is effective against several periodontitis-causing bacteria.[4] However, the systemic use of antibiotic drug at high dosage may cause several side effects including hypersensitivity, gastrointestinal intolerance, and also a chance of development of bacterial resistance.[5,6] Furthermore, it has been reported that systemic administration of the drug often requires high concentrations to maintain a therapeutic effect because of the dilution effect that can cause issues with maintaining an effective dose at the disease site.[7] Thus, there is an interest in a controlled drug delivery system that can overcome some of the problems of systemic administration and enhance the therapeutic efficacy of a given drug with minimal side effects.[8–12]

Numerous controlled release drug delivery systems have been investigated to provide drug release over extended periods of time.[13,14] Recently, biodegradable polymeric microspheres encapsulating antibiotic drugs have received much attention for periodontal applications.[15,16] Several studies aimed at optimizing the drug-release behavior from drug-loaded microspheres prepared via double emulsion-solvent evaporation methods.[17,18] The drug released from polymeric microspheres made via water-in-oil-in-water (W1/O/W2) emulsion is governed by various properties including biodegradability of core polymer, solubility of the drug, and type of polymer stabilizer. Several biodegradable polyesters, such as polylactic acid (PLA), polylactic-co-glycolic acid (PLGA), polycaprolactone (PCL), and polyhydroxyalkanoates (PHAs) have been used for microspheres preparation and controlled delivery of drugs.[19–23]

Additional Supporting Information may be found in the online version of this article.

Among biodegradable polyesters, PHAs have received increasing attention in the biomedical field due to their superior properties, including tunable mechanical properties, biocompatibility, and biodegradability.[24,25] PHAs are a class of aliphatic polyesters synthesized as inclusion bodies in various bacteria and used as carbon and energy storage deposits under nutrient-limited conditions.[26,27] Bacterial PHAs are typically produced with high molecular weight (M_w; i.e., 10^5-10^6 g/mol) and low polydispersity index. They are highly biodegradable with excellent human biocompatibility.[28] In addition, they can be produced with different side chain compositions, which alter their physical properties and render them useful for a wide range of applications.[29] The most common type of PHAs in nature is polyhydroxybutyrate (PHB), which is relatively stiff and brittle.[30] However, incorporation of hydroxyvalerate (HV) monomers along with hydroxybutyrate (HB) forms a copolymer known as PHB-co-hydroxyvalerate (P(HB-HV)), which make the PHA more flexible and can change the polymer degradation rate.[31,32] Both PHB and P(HB-HV) have been successfully demonstrated for preparation of drug-encapsulated microspheres, where the drug release behavior was influenced by properties, such as HV content.[33–37] Coimbra *et al.*[33] prepared polymeric microspheres from biodegradable P(HB-6HV) copolymers and showed controlled release of a non-steroidal anti-inflammatory drug, flurbiprofen. Another drug release study encapsulated sulfamethizole into PHB and P(HB-HV) copolymeric microparticles coated with L-poly(lactic acid).[34] Sendil *et al.*[35] incorporated TC into P(HB-HV) microspheres and demonstrated the extended release of the drug both in its acidic and neutral forms for about 26 h. Recently P(HB-9.8%HV) microparticles were demonstrated for delivery of another antibiotic rifampicin.[36] Therefore, drug delivery microspheres prepared from various PHAs allow tuning of polymer properties and enable controlled release of a variety of antibiotics.[38]

In addition to the core polymer composition, the influence of polymer stabilizer that generates a shell around the hydrophobic polymeric microspheres is another parameter to consider when formulating controlled release drug delivery systems. Polyvinyl alcohol (PVA), a nonionic amphiphilic polymer obtained from hydrolysis of polyvinyl acetate, is one of the most commonly used stabilizers in the preparation of micro- and nanoparticles for controlled drug delivery.[39–42] Several studies also showed that the presence of PVA stabilizer could influence many important properties of drug-loaded polymeric microspheres including particle size, zeta potential, polydispersity index, drug loading, entrapment efficiency, and drug release profile.[20,39,43,44] However, there is still limited information regarding the influence of the PVA stabilizer on the drug release profile PHA microspheres.[40–47] Therefore, it is of interest to study the influence of types of PHA core and also the impact of PVA shell stabilizer on general characteristics of formulated microspheres as well as the loading and release profiles of antibiotic drugs for potential application in periodontitis treatment.

In this study, we prepared and characterized polymeric microspheres of bacterially derived PHAs and investigated their ability to encapsulate and release TC for potential use in dental applications. We selected TC as it is currently used to combat periodontal pathogens in the treatment of periodontal disease and represents a good hydrophilic drug model for polymeric encapsulation studies.[35] Four types of bacterially derived PHAs, such as PHB, P(HB-5HV), P(HB-12HV), and P(HB-50HV), were investigated in this study. TC was encapsulated into polymeric microspheres via a W1/O/W2 double emulsion-solvent evaporation method using PVA stabilizer with two different M_ws (i.e., low M_w PVA and high M_w PVA). The influence of PHA as core polymer and the effect of PVA as stabilizer polymer on size, drug loading, drug entrapment efficiency, drug release profile, and drug release mechanism of TC-loaded microspheres were investigated. The antibacterial activity of TC-loaded microspheres was evaluated against two common strains of periodontal-causing bacteria; *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. The results suggest the potential application of PHA microspheres, especially PHB, for controlled slow-release delivery system of hydrophilic TC for the treatment of periodontal disease. This study also indicates that the polymeric microsphere structure and drug release characteristics could be modulated by changing PHA composition and also by using different M_ws of the PVA stabilizer.

EXPERIMENTAL

Materials

Bacterially derived PHB ($M_w = 3.50 \times 10^5$ g/mol), P(HB-HV) with HV content 5 wt % P(HB-5HV) ($M_w = 2.04 \times 10^5$ g/mol) and 12 wt % P(HB-12HV) ($M_w = 2.50 \times 10^5$ g/mol), PVA with 87–89% degree of hydrolysis including low M_w PVA (M_w = 13,000–23,000 g/mol) and high M_w PVA (M_w = 85,000–124,000 g/mol), and TC:HCl were obtained from Sigma-Aldrich (St. Louis, MO). Chloroform, methanol, and ethanol were purchased from RCI Labscan (Bangkok, Thailand). Phosphate-buffered saline (PBS, pH 7.4) solution was prepared using chemicals from Merck Chemicals (Darmstadt, Germany). Brain heart infusion (BHI), tryptic soy broth (TSB), and blood agar culture medium were supplied by Difco Microbiology (Detroit, MI). All other chemicals and solvents were of analytical grade and used without further purification.

Production and Characterization of P(HB-50HV)

P(HB-50HV) was prepared by bacterial cultivation "in house" as previously described.[48,49] Briefly, *Cupriavidus necator* H16 was used for P(HB-50HV) polymer synthesis by two-stage cultivation. Sodium valerate was added to mineral salts medium as a carbon substrate in order to promote the biosynthesis of P(HB-50HV). Polymer-accumulating cells were centrifuged and washed twice with PBS and subsequently lyophilized for 24 h (Freeze zone plus 6; Labconco). The methanolysis in chloroform of the lyophilized cells in the presence of 85% (v/v) methanol and 15% (v/v) sulfuric acid was carried out[50] prior to determining polymer composition through gas chromatography analysis (Model 6890 plus; Agilent Technology) equipped with HP-INNOWAX 30.0 m, 0.25 mm, 0.25 μm capillary columns, and flame deionization detector. P(HB-50HV) copolymer was purified by an established chloroform solvent extraction process following a previously described method.[51] The polymer was precipitated with a 10-fold volume of −20°C cold methanol. The P(HB-50HV) separated from methanol during stirring

Table I. Characteristics of Tetracycline-Loaded PHA Microspheres

Types of PVA	Types of core polyester	Particle size (μm)	Drug loading (%)	Entrapment efficiency (%)	Residual PVA (%)
Low M_w	PHB	35.87 ± 9.45[c]	13.53 ± 0.32[b]	29.47 ± 0.69[b]	2.43 ± 0.33[b]
	P(HB-5HV)	20.00 ± 5.17[c]	13.82 ± 0.29[b]	29.73 ± 0.67[b]	1.81 ± 0.46[b]
	P(HB-12HV)	9.50 ± 0.59[b]	13.04 ± 0.29[b]	30.45 ± 0.67[b]	1.49 ± 0.07[b]
	P(HB-50HV)	329.90 ± 8.71[a] (nm)	3.80 ± 0.58[a]	8.47 ± 1.48[a]	0.89 ± 0.45[a]
High M_w	PHB	9.16 ± 1.16[c]	7.97 ± 1.03[b]	20.81 ± 2.74[b]	1.39 ± 0.52[b]
	P(HB-5HV)	10.00 ± 1.22[c]	10.56 ± 0.85[c]	26.04 ± 1.92[b]	1.22 ± 0.08[b]
	P(HB-12HV)	5.25 ± 2.16[b]	4.55 ± 0.07[a]	12.43 ± 0.20[a]	0.65 ± 0.17[a]
	P(HB-50HV)	263.50 ± 12.43[a] (nm)	3.02 ± 0.63[a]	8.22 ± 1.74[a]	0.64 ± 0.15[a]

Low M_w PVA: 13,000–23,000 g/mol and high M_w PVA: 85,000–124,000 g/mol. Each value is mean ± SD of triplicate (n = 3). Different small letters (a–c) within same column for each type of PVA indicate significant difference at $p \leq 0.05$.

(15 min, 1,500 rpm), and the precipitated polymer was recovered by filtration and left to dry in a chemical fume hood for at least 24 h. The % purity and the composition of the %HV monomer content of the purified polymer were also reconfirmed with ^{1}H NMR (Bruker Avance-500 MHz spectrometer, Bruker, Germany). The M_w and polydispersity index of P(HB-50HV) were determined by gel permeation chromatography to be 1.69 × 10^6 g/mol and 1.48, respectively as described in the previous study.[52]

Preparation of TC-Loaded Polymeric Microspheres

Various formulations of polymeric microspheres, made from four different core polymeric PHAs, such as PHB, P(HB-5HV), P(HB-12HV), and P(HB-50HV) (Table I), were prepared by a modified W1/O/W2 double emulsion-solvent evaporation method as previously described.[53] Briefly, 500 μL of TC solution with an equivalent to 40% (w/w) initial drug loading was prepared in water and added into 5 mL of 10% (w/v) chloroform solution of the various polymers. Then, the polymer solution was homogenized using a probe ultrasonicator (Bandelin Sonopuls, Germany) at 90 W for 3 min to form a stable W1/O solution. The primary W1/O emulsion was then mixed with 20 mL of 1% (w/v) PVA solution having different M_ws (i.e., low M_w PVA: 13,000–23,000 g/mol or high M_w PVA: 85,000–124,000 g/mol) and sonicated at 25 W for 1 min, to prepare the W1/O/W2 double emulsion. Subsequently, the emulsion was added to 20 mL distilled water and stirred at 1,200 rpm for 4 h at room temperature to allow chloroform (i.e., 5 mL) to evaporate. Later, the TC-loaded polymeric microspheres were collected by centrifugation at 4,024g for 20 min. The microspheres were washed by resuspending and centrifuging twice with distilled water in order to remove nonencapsulated TC and loosely bound PVA. Samples were then then lyophilized for 24 h and stored in a −20°C freezer (SF-C1497 freezer; Sanyo, Japan), if not used immediately.

Characterization of Particle Size and Morphology

Particle size and distribution of TC-loaded polymeric microspheres were determined by random counting of 100 particles from each microsphere formulation under bright-field microscope (Olympus BX53M; Olympus Ltd.). The particle size and size distribution were determined by converting the particle area to particle diameter using ImageJ and then analyzed the data in Origin Pro 9 (OriginLab Corporation, MA). Surface and morphological properties of microspheres were analyzed by scanning electron microscopy (SEM) (JSM 6400; Jeol, Japan). Prior to SEM imaging, the samples were sputter coated with a thin layer (∼10 nm) of gold.

Determination of Drug Loading and Encapsulation Efficiency

To determine the % drug loading and % encapsulation efficiency of TC-loaded microspheres, an accurately weighed sample of freeze-dried microspheres was added to 1 mL $CHCl_3$ and mixed by vortex mixer until all microspheres were dissolved. Then, 5 mL of distilled water was added and continuously mixed for 20 min to extract the drug into water. The amount of drug present in the aqueous phase was analyzed by measuring the absorption at 290 nm using a spectrophotometer (Helios Alpha; Thermo Scientific). The TC content of each microsphere formulation was calculated using a standard curve of the free drug dissolved in distilled water measured at the same wavelength. Percentages of drug loading and encapsulation efficiency were then calculated with the following equations:

$$\text{Drug loading (\%)} = \frac{\text{Amount of TC in microspheres (mg)}}{\text{Amount of microspheres (mg)}} \times 100 \quad (1)$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Actual drug loading (\%)}}{\text{Theoretical drug loading (\%)}} \times 100 \quad (2)$$

In Vitro Release of TC and Drug Release Kinetics

The time-release of TC from PHA microspheres was determined as follows: 10 mg of lyophilized TC-loaded PHA microspheres was immersed in 10 mL PBS solution pH 7.4 at 37°C and kept in the dark. At predetermined time intervals, 1 mL samples of the PBS medium were collected and then replaced with an equal amount of fresh PBS. The released TC in each sample was determined by reading the absorbance at 290 nm and comparing to a standard curve of pure TC in PBS pH 7.4.

In order to characterize the drug release kinetics and mechanism of drug release from TC-loaded PHA microspheres, the data obtained from *in vitro* drug release studies were analyzed

using zero-order kinetic equation, first-order kinetic equation, and Korsmeyer–Peppas kinetic model.

The zero-order kinetic equation describes a system where the drug release rate is concentration independent.[54] Zero-order kinetic model is described in the following equation:

$$C = k_0 t \quad (3)$$

where C is the cumulative concentration of drug released, k_0 is the zero-order rate constant expressed in units of concentration per time, and t is the time in hours.

The first-order kinetic equation describes a system where the drug release rate is depending on the concentration remaining in the polymeric network.[54] First-order kinetic model determines as log cumulative percent drug remaining versus time as the following equation:

$$\log C = \log C_0 - kt/2.303 \quad (4)$$

where C_0 is the initial concentration of drug and k is the first-order constant.

The Korsmeyer–Pappas model was used to determine whether the drug release mechanism is influenced by both diffusion and dissolution.[55] This model determines as log cumulative percent drug remaining versus log time, describing the first 60% drug release from a polymeric system according to the following equation:

$$M_t/M_\infty = k_{KP} t^n \quad (5)$$

where M_t/M_∞ is the fraction of drug released at time t, k_{KP} is the rate constant, n is the diffusional exponent, and t is the time point. The values of n were determined from the slope and intercept of a straight line fitted to the data. The n value can be used to characterize different release mechanisms, where in the case of Fickian release (i.e. diffusion-controlled release) from spherical matrices or cylindrical tablets, the value of n is $\leq$0.43, whereas in case-II transport (i.e., relaxation-controlled release), n is $\geq$0.85. The non-Fickian release (i.e., anomalous transport) of drugs occurs when the values of n fall within 0.43 and 0.85.[55,56]

Determination of PVA Content Associated with Microspheres

The amount of residual PVA associated with each TC-loaded PHA microsphere preparation was determined by a colorimetric method based on the formation of a colored complex between two adjacent hydroxyl groups of PVA and iodine molecule according to a previously described protocol.[43] Briefly, 10 mg of each TC-loaded microspheres was treated with 2 mL of 0.5 *M* NaOH for 45 min at 60°C. The mixture was then neutralized with 9 mL of 1 *M* HCl and 5 mL of distilled water was added. Thereafter, 3 mL of a 0.65 *M* solution of boric acid, 0.5 mL of a solution of I_2/KI (0.05/0.15 *M*) and 1.5 mL of distilled water were added to form a blue-colored complex. After 15 min incubation, the absorbance at 690 nm was measured and the residual PVA content calculated from a standard curve as percentage of residual PVA associated with TC-loaded PHA microspheres.

Antimicrobial Activity of Microspheres

The antibacterial activity of selected TC-loaded PHA microspheres was studied using a standard broth dilution technique as previously described.[57] Briefly, the TC-loaded polymeric microsphere formulation that showed the most desirable characteristics (such as high drug loading and slow drug release profile) was tested for its antibacterial property against two oral periodontal-causing bacteria *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* Y4. *P. gingivalis* was cultured in BHI broth and incubated for 3 days under anaerobic environment with an atmosphere of 5% H_2, 10% CO_2, and 85% N_2 at 37°C. *A. actinomycetemcomitans* was cultured under CO_2 incubation at 37°C for 3 days in TSB. After incubation, the optical density of the bacterial inoculum for each strain was determined using a spectrophotometer and adjusted to be equivalent to 10^8 colony-forming unit (CFU)/mL (based on a CFU/mL vs. turbidity curve). Then, 10 mg of TC-loaded microspheres was added into 10 mL of bacterial broth media. The cultures were incubated at 37°C for 3 days and the reduction in bacterial count overtime (every 2 h) was determined using a colony counting assay on blood agar plates.[58] The antibacterial activity of TC-loaded microspheres, free TC with equivalent mass added (positive control), and unloaded microspheres (negative control) were plotted a on a log CFU/mL scale where comparisons were made on the percent reduction of log CFU/mL with respect to non-treated control.

Statistical Analysis

The obtained data were compared and evaluated by employing the analysis of variance. Differences of $p \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Particle Size and Morphological Characteristics of Microspheres

The TC-loaded PHA microspheres were prepared by the W1/O/W2 double emulsion and solvent evaporation technique using two different M_w PVA stabilizers as presented in Table I, TC-loaded PHAs:low M_w PVA microspheres produced average particle size ranging from 9.50 ± 0.59 to 35.87 ± 9.45 μm. It was found that the mean particle size decreased with the increase in %HV content, especially the core P(HB-HV) copolymer with 5 and 12% HV. In fact, when the P(HB-50HV) copolymer was used, it resulted in sub-micron particles with average size around 329.90 ± 8.71 nm. Similar results were also observed with TC-loaded PHAs:high M_w PVA microspheres, which showed similar trends in particle size (Table I), ranging from 5.25 ± 2.16 to 10.00 ± 1.22 μm for PHB and P(HB-HV) with 5 and 12% HV, while the P(HB-50HV) yielded submicron particles with average size around 263.50 ± 12.43 nm. The drastic decrease in particle size when using P(HB-50HV) might be due to the relative lower viscosity of the organic P(HB-50HV) solution used in the emulsification step compared to PHB and other P(HB-HV) polymers with lower %HV content.[59] We noted that using a polymer with higher %HV ratio reduced the viscosity of the organic solution (data not shown). Several studies have reported similar findings where well dispersed emulsion droplets and smaller size particles could form in the PVA aqueous phase when using a lower viscosity organic polymer solution.[60,61] Our findings are also in accordance with a study performed by Wang *et al.*[61] that found increasing HV content

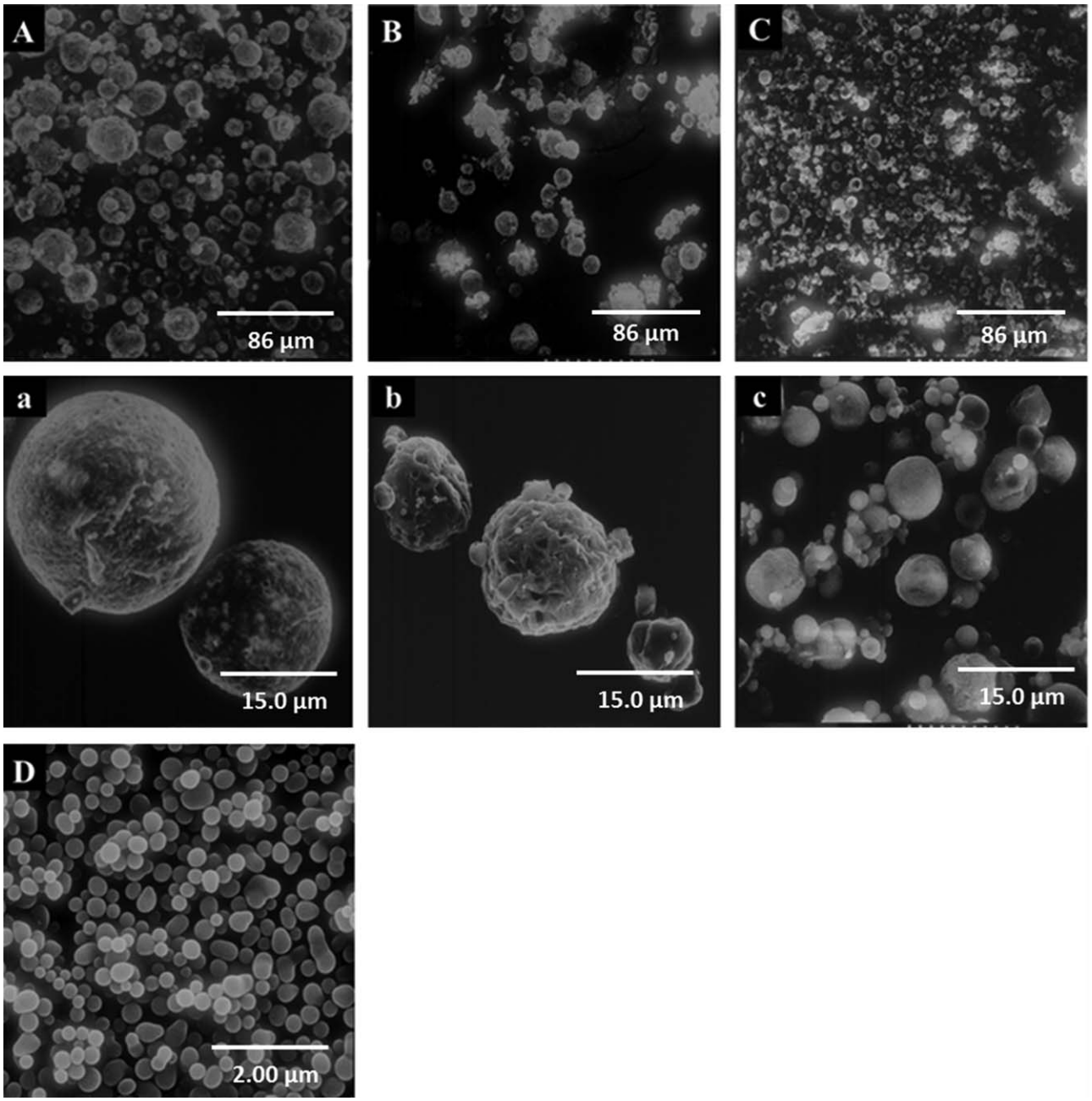

Figure 1. Scanning electron micrographs of the TC-loaded PHAs:low M_w PVA microsphere structures; (A) PHB, (a) close-up view of PHB, (B) P(HB-5HV), (b) close-up view of P(HB-5HV), (C) P(HB-12HV), (c) close-up view of P(HB-12HV), and (D) P(HB-50HV).

in P(HB-HV) copolymer decreased the average size and size distribution of ibuprofen-loaded P(HB-HV) microparticles. This was attributed to more efficient dispersion of the organic phase in the aqueous medium.

Morphological characteristics of TC-loaded PHA microspheres were analyzed using SEM. The TC-loaded PHAs:low M_w PVA and TC-loaded PHAs:high M_w PVA microspheres exhibited a fairly homogenous size distribution, displaying a slightly rough and spherical morphology as shown in Figures 1 and 2, respectively. All TC-loaded PHAs microspheres exhibited a slightly rough surface morphology with apparent pores. Generally, an increase in %HV content of the core polymer appear to lead to more spherical particles with a smoother and less porous surface morphology. This is attributed to the less crystalline characteristic of the copolymer compared to PHB homopolymer microspheres.[33] The increase in porosity and roughness on the surface of PHB microspheres has been associated with a faster rate of crystallization and high crystallinity of PHB polymer in aqueous solution.[35,38] In comparison with the P(HB-50HV) copolymers, PHB may have a more porous structure as a result of the hardening of the surface, which prevents particles from shrinking.[63] Grillo *et al.*[37] showed that P(HB-HV) microspheres,

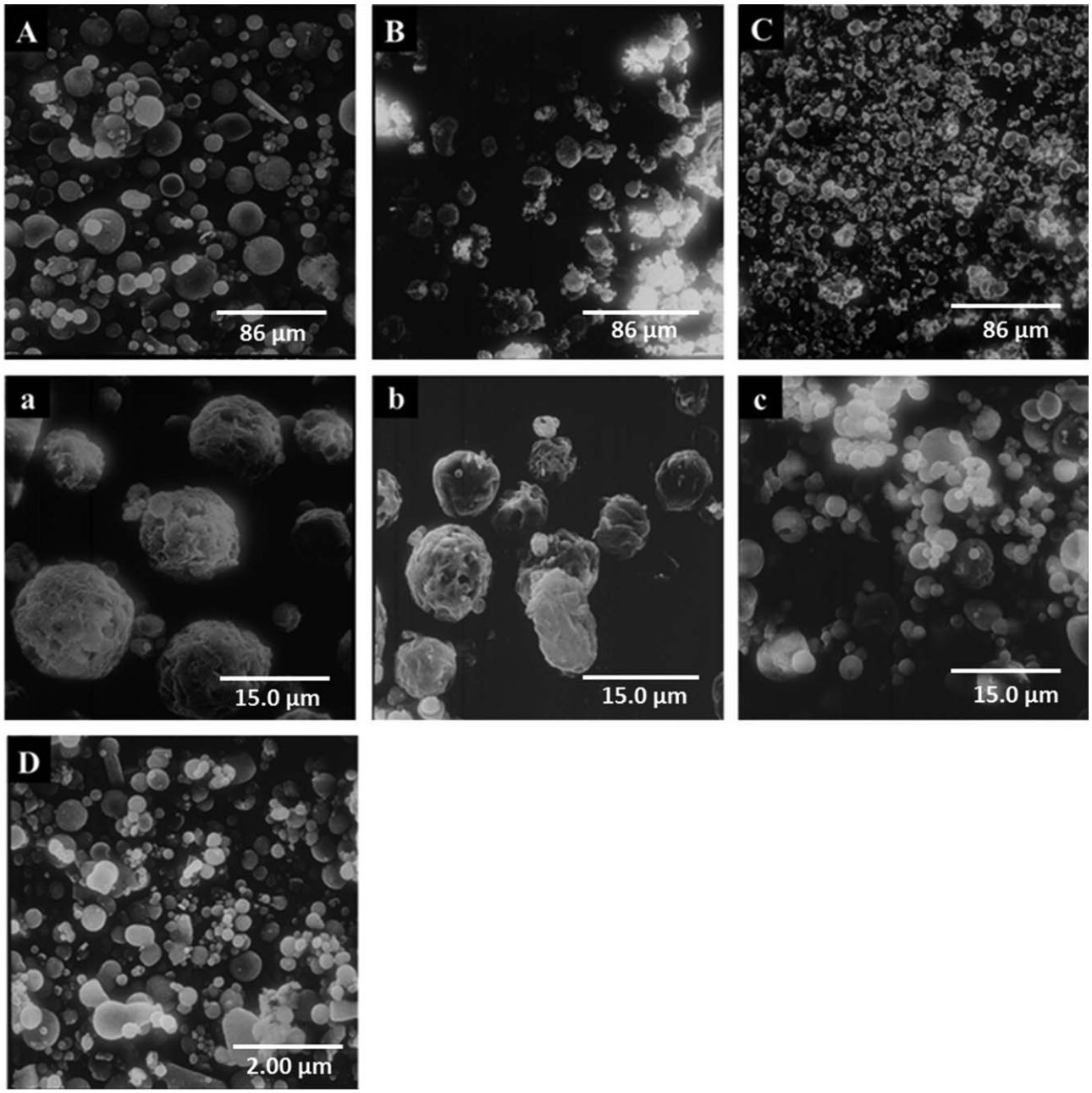

Figure 2. Scanning electron micrographs of TC-loaded PHAs:high M_w PVA microsphere structures; (A) PHB, (a) close-up view of PHB, (B) P(HB-5HV), (b) close-up view of P(HB-5HV), (C) P(HB-12HV), (c) close-up view of P(HB-12HV), and (D) P(HB-50HV).

prepared using a similar technique to ours, exhibited a spherical shape with a smooth surface and no obvious pore formation on the surface.

Analysis of Surface-Associated PVA of Microspheres

When comparing the effect of using low M_w and high M_w PVA on the particle size, there was a significant decrease in particle size for each type of PHA microspheres when high M_w PVA was used in the emulsification process. One reason might be that different amount of PVA is absorbed onto the surface of PHA microspheres, here referred to as % residual PVA. The amount of either low M_w or high M_w PVA associated with different types of TC-loaded PHA microspheres was quantified and shown in Table I. For all types of PHA microspheres, the ones prepared from high M_w PVA had a slightly lower % residual PVA compared to the low M_w PVA preparations. The highest amount of residual PVA absorption was found on the PHB:low M_w PVA (2.43 ± 0.33%, w/w), while the least amount of adsorbed PVA was found on the P(HB-50HV):high M_w PVA (0.64 ± 0.15%, w/w). Interestingly, we found that the amount of residual PVA for both low MW and high M_w PVA appeared to decrease with increasing %HV content in the P(HB-HV) microspheres.

Studies have showed that the amount of PVA associated to hydrophobic microspheres is likely influenced by the M_w of PVA used during microsphere formulation.[44] Typically, surface

adsorption of PVA involves conformation rearrangements of PVA chains at the emulsion interface.[64] The acetate groups of PVA is known to interact with the hydrophobic particles, while the hydrophilic alcohol groups of PVA help to reduce the surface energy by interacting with the aqueous phase.[65] Buttini *et al.*[44] found that the absorption of smaller PVA molecules (i.e., lower M_w PVA) onto the surface of hydrophobic particles occurred at faster rate than larger PVA molecules (i.e., high M_w PVA) due to less restriction in their conformational chain rearrangement. They also suggested that low M_w PVA might have multilayer absorption to the hydrophobic particle surface, thus leading to higher surface concentration of PVA on the particles.[43,44] Our results here appear to indicate a similar behavior. In the case of high M_w PVA polymers, a stronger surface binding could lead to a more stable interface that facilitates formation of smaller particles when compared to low M_w PVA.

Characterization of Drug Loading and Encapsulation Efficiency

The drug loading and entrapment efficiency are important characteristics of any drug-encapsulated microsphere formulation. To determine the effect of various types of PHA core polymers as well as the effect of PVA M_w surface stabilizers on drug loading and entrapment efficiency, we fixed the theoretical loading amount of TC in all formulations at 40% (w/w). This was based on preliminary screening results using various % theoretical loading (data not shown) indicating this concentration offered a good encapsulation efficiency overall.

The drug loading and encapsulation efficiency of various PHA microsphere formulations are summarized in Table I. The TC loading and encapsulation efficiency in PHAs:low M_w PVA microspheres were around 13–14 and 29–30%, respectively, for PHB, P(HB-5HV), and P(HB-12HV) microspheres, whereas P(HB-50HV) showed a significantly lower TC loading at 3.80% and encapsulation efficiency at 8.47%. It appears that increasing HV content from 5 to 12% in the core polymer is not enough to have a significant effect on the drug loading and encapsulation efficiency. The decrease in drug loading and encapsulation efficiency of P(HB-50HV) could be attributed to the smaller particle size in sub-micron rage. Previous studies have demonstrated that both drug loading and encapsulation efficiency could be reduced when decreasing particle size.[19,66] Another explanation might be that P(HB-HV) polymers generally exhibits more amorphous (i.e., less crystalline) structure than PHB homopolymer due to the presence of hydroxyvalerate side chains,[67,68] thus allowing the polymer chains to be loosely packed during solidification in the solvent evaporation step.[69] As a result, it is possible that the hydrophilic TC molecules could leak from the internal polymeric phase into the second aqueous phase (W2) during the preparation of the second emulsion, lowering the drug loading and drug entrapment efficiency. Similar observations has been noted by Sendil *et al.*[35] In another study, Gangrade and Price[70] reported that a progesterone steroid hormone had less drug loading in P(HB-24HV) microspheres compared to PHB and P(HB-9HV).

Besides the effect of %HV content in core polymer, the influence of PVA M_w stabilizer on the drug loading and entrapment efficiency was also investigated. As shown in Table I, the amount of TC loading and entrapment efficiency were reduced from 14% (low M_w PVA) to around 5–11% drug loading (high M_w PVA) and from 30% (low M_w PVA) to around 12–26% entrapment efficiency (high M_w PVA) for PHB, P(HB-5HV), and P(HB-12HV) microspheres, respectively. Similar trends were observed for the P(HB-50HV) formulations where the drug loading was reduced with approximately 20% when using high M_w PVA. The reduced loading for high M_w PVA could be due to several reasons. First, Buttini *et al.*[44] reported that high M_w PVA stabilizer had slower adsorption rate onto the surface of hydrophobic particles than low M_w PVA. As a result, the high M_w PVA often led to slower particle formation, causing hydrophilic drugs like TC to leach out during drug entrapment process.[71] Another explanation might be related to the reduction in particles size when using high M_w PVA as argued above for the differences in drug loading for the different core polymers.

Drug Release Characteristics of Microspheres

It is of interest to study whether the formulation parameters, such as the type of core PHA polymer, and M_w of PVA stabilizer can influence the drug release from biodegradable microspheres. For the purpose of this study, extended drug release characteristics were considered favorable since the critical treatment period for periodontitis is typically between 7 and 10 days.[72]

Effect of PHAs on *In Vitro* Drug Release Profile. Drug release studies were performed *in vitro* in PBS buffer pH 7.4 at 37°C over 7 days for all TC-loaded PHA microspheres. Figure 3 shows the release profiles of TC from different PHAs:low M_w PVA [Figure 3(A,B)] or PHAs:high M_w PVA [Figure 3(C,D)]. The release profiles of TC from various core PHA microspheres showed similar patterns for both low M_w and high M_w PVA. PHB microspheres had the lowest initial burst release with 13% TC over the first hour, followed by a cumulative release upto 80% at day 3, and a continued slow release over 7 days. In contrast, all P(HB-HV) microspheres with 5, 12, and 50% HV content showed a rapid inital release at around 20% in the first hour, where subsequently >80% of the drug content was released within the first day. In general, TC showed a burst release from PHA microspheres, followed by a lag phase exhibiting a slower release behavior with a final period of steady release. The initial burst release of TC could be asscociated with fast difussion of drug located at the microsphere's surface.[73] This initial phase was followed by a slower, more controlled release period where the drug diffuse from the microsphere core into the release buffer.

Our results showed that increasing %HV content of P(HB-HV) microspheres led to faster release of TC, which could be attributed to the more amorphous structure of the P(HB-HV) microspheres. It is known that higher %HV component in PHA copolymers results in a more amorphous structure.[35] These copolymers have a higher tendency to take up water, which relax the polymer matrix and facilitate the drug diffusion.[74] Results from DSC analysis shown in Table S1 and Figure S1, confirmed that increasing %HV content in PHA microspheres

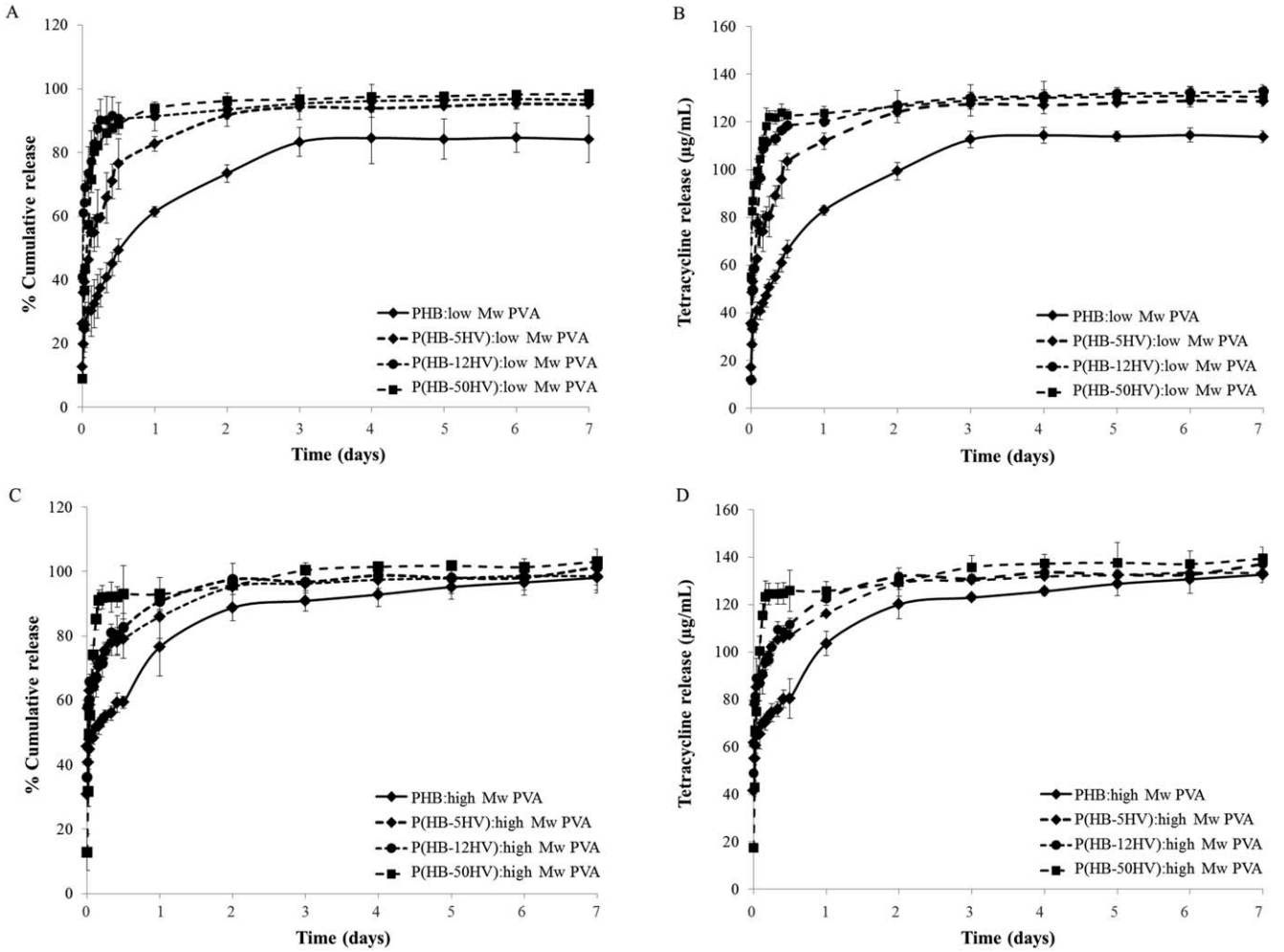

Figure 3. *In vitro* release profiles of microspheres prepared from the various types of PHA core polymer; (A) percentages of TC release from PHAs:low M_w PVA microspheres, (B) concentrations of TC release from PHAs:low M_w PVA microspheres, (C) percentages of TC release from PHAs:high M_w PVA microspheres, and (D) concentrations of TC release from PHAs:high M_w PVA microspheres.

led to a significant decrease in the enthalpy of melting and melting temperature of TC-loaded PHA microspheres from 173.83 to 82.10°C, suggesting reduced crystallinity. Previous studies reported similar trends where PHA microspheres with higher %HV content had faster drug release rates compared to more crystalline particles.[75,76] Another factor to consider is the smaller particle size of P(HB-HV) microspheres. The reduction in particle size would increase the particle surface area exposed to water, allowing more water incursion and faster drug release.[77,78]

Effect of PVA on *In Vitro* Drug Release Profile. PVA is a commonly used particle stabilizer and another component to consider in hydrophobic polymer-based drug delivery systems.[39,44] The role of surface adsorbed PVA has been investigated and observed to play a critical role in cellular internalization of micro-and nanoparticles.[43,79] In addition, PVA has also been demonstrated to influence the release of drug and bioactive molecules from various polymeric microspheres.[44,80] Thus, we were interested in investigating the influence of PVA M_w on the drug release behavior of our TC-loaded PHA microspheres.

The release profiles of TC from various PHA microspheres prepared from different M_w PVA as stabilizer (i.e., low M_w 13,000–23,000 g/mol vs. high M_w 85,000–124,000 g/mol, both with the same degree of hydrolysis in a range of 87–90%) are shown in Figure 4(A–D). Both PVA systems exhibited a similar biphasic release pattern; however, the PHAs:high M_w PVA system had a more pronounced initial burst release and a faster cummulative TC release behavior. During the first two hours, PHAs:low M_w PVA displayed an initial burst release of 15–20%, which were lower than PHAs:high M_w PVA. The TC released from all formulations gradually increased to 40–80% within 12 h.

The lower initial burst release from PHAs:low M_w PVA microspheres could be a result of higher % residual PVA associated with the particle surface. The residual PVA could influence the access of water and diffusion of entrapped TC from the internal polymer matrix, resulting in an apparent slower release. Landry *et al.*[81] reported a similar finding where increased concentration of PVA on the surface of polylactide (PLA) particles lead to a slower drug release rate that was attributed to reduced access of water molecules, thus slowing the hydrolytic degradation of PLA.

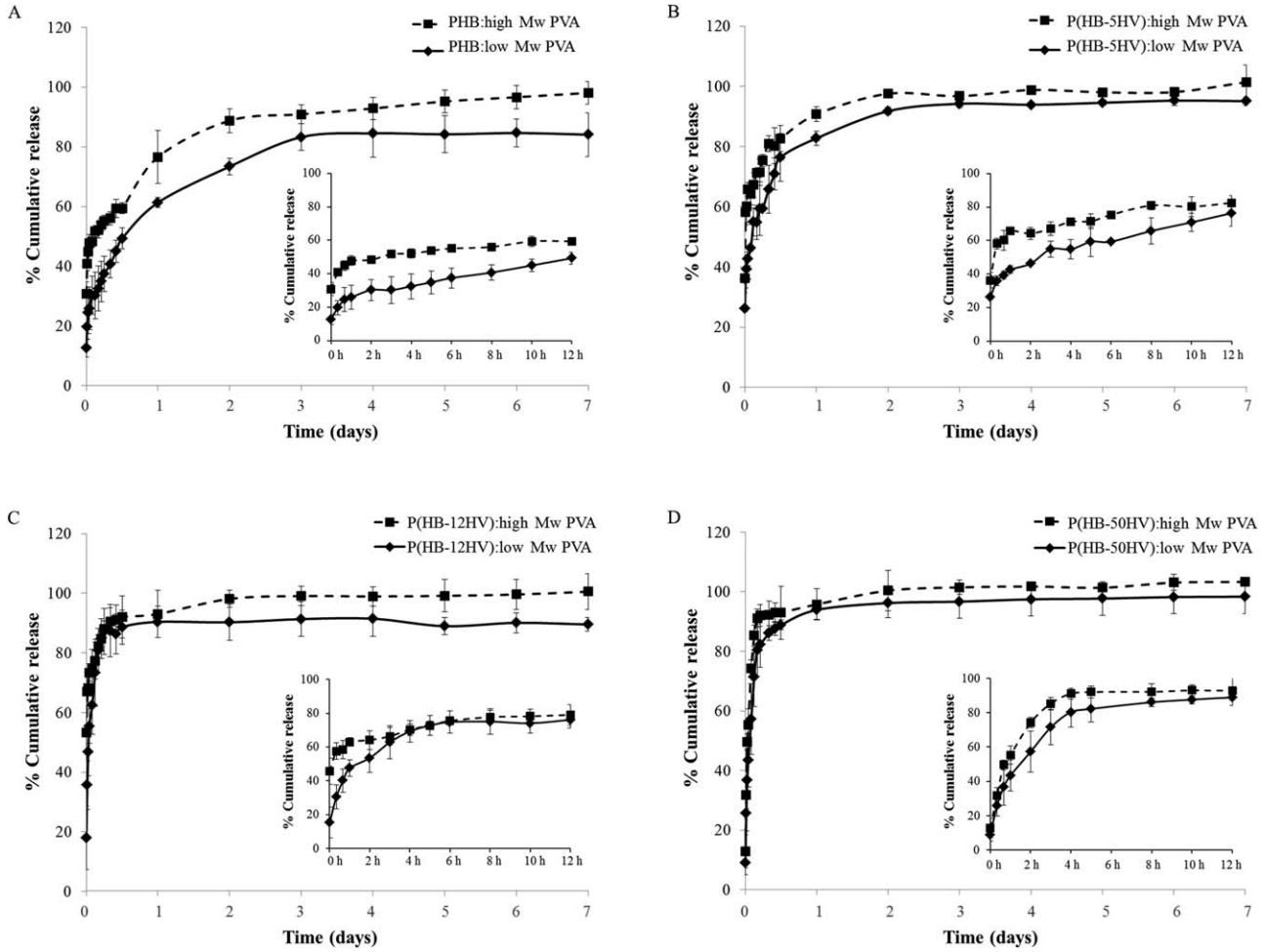

Figure 4. *In vitro* release profiles of microspheres prepared with different M_w of PVA stabilizer; (A) PHB, (B) P(HB-5HV), (C) P(HB-12HV), and (D) P(HB-50HV). The insert shows the initial release behavior within the first 12 h from PHA microspheres.

Release Kinetics of Encapsulated TC

Comparing the two PVA systems, PHAs:low M_w PVA exhibited higher drug loading, as well as slower drug release behavior, both which are considered desirable for periodontitis drug delivery applications. Thus, the drug release kinetics of all four TC-loaded PHAs formulated with low M_w PVA stabilizer was further characterized in more detail.

The TC release data were fitted to zero-order and first-order kinetic models in order to analyze the release behaviour. To evaluate the release mechanism, the data were fitted to the Korsmeyer–Peppas model using nonlinear regression fitting. The kinetic rate constants (k) and release exponent (n) of each model were calculated by linear regression analysis. Coefficients of correlation (R^2) were used to assess the goodness of the fit. Based on the correlation coefficient values, the most appropriate model was selected to describe the release behavior of the drug. The fitted values of the kinetic rate constant (k), the correlation coefficient (R^2), and the release exponent (n) are shown in Table II.

Table II. Correlation Coefficient (R^2), Release Rate Constants (k), and Diffusion Coefficient (n) of the Kinetic Models Applied to the Release of Tetracycline from PHAs:Low M_w PVA Microspheres

Types of microsphere	Zero-order		First-order		Korsmeyer–Peppas	
	R^2	k_0 (1/h)	R^2	k_1 (1/h)	R^2	N
PHB	0.8853	1.8393	0.9535	−0.0137	0.9580	0.2475[a]
P(HB-5HV)	0.8468	4.2280	0.8973	−0.0342	0.8742	0.1058[a]
P(HB-12HV)	0.8235	17.8060	0.8793	−0.1251	1.0000	0.1717[a]
P(HB-50HV)	0.9073	22.6770	0.9662	−0.1586	1.0000	0.4049[a]

[a] n value ≤0.43 indicates Fickian diffusion control release.

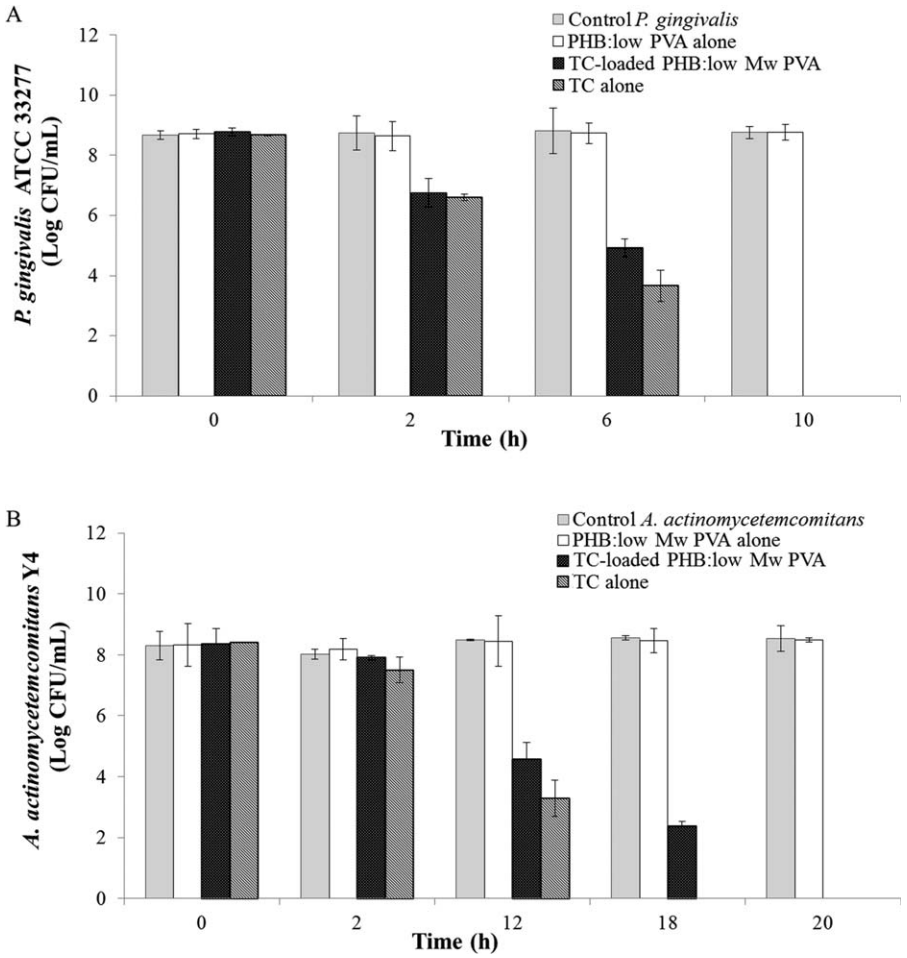

Figure 5. *In vitro* antibacterial activity of TC-loaded PHB:low M_w PVA microspheres against periodontal pathogenic bacteria; (A) *Porphyromonas gingivalis* and (B) *Actinobacillus actinomycetemcomitans*.

All four PHA microsphere formulations with low M_w PVA had better fits to the first-order model with R^2 values close to 1. Thus, the TC release was dependent on the drug concentration in the microsphere matrix. In addition, the k values from the first-order model also suggests the order of TC release rate from fast to slow as follows: P(HB-50HV) > P(HB-12HV) > P(HB-5HV) > PHB. Fitting to the Korsmeyer–Peppas model gave correlation coefficients above 0.87. Considering the Korsmeyer–Peppas equation, the n values for all four PHA microsphere formulations were $\leq$0.43, suggesting that the TC released from PHA microspheres follows a Fickian diffusion-based drug release mechanism.[55]

Antibacterial Activity of Microspheres

Among the eight formulations, PHB:low M_w PVA microspheres had the highest drug loading as well as the slowest TC release over 7 days. We considered this to be the most suitable formulation for the potenital treatment of periodontal disease and thus it was selected for futher antibacterial activity studies.

Antibacterial assays using *P. gingivalis* and *A. actinomycetemcomitans* were performed where the bacterial susceptibility to TC in microparticle fomulations was evaluated by comparing the log reduction of viable cells. The initial bacterial concentration used in our experiment was 10^8 CFU/mL, a challenge concentration eqivalent to the bacterial concentration associated with severe periodontal infection.[82] The susceptibility of *P. gingivalis* and *A. actinomycetemcomitans* to TC where the bacterial population is inhibited to 90% (minimum inhibitory concentration "MIC_{90}") is 0.5 and 8 μg/mL, respectively.[83]

The results of the antibacterial activitiy studies are given in Figure 5. The TC-loaded PHB microspheres with low M_w PVA exhibited strong antibacterial potential with 85% reduction of *P. gingivalis* within the first 2 h (corresponding to a TC released concentration of 40.90 μg/mL). The amount of TC released from the microspheres during the first 12 h (66.72 μg/mL) reached sufficient concentration to eradicate all *P. gingivalis* bacteria, whereas it only reduced the bacterial viability to less than 10^5 CFU/mL for *A. actinomycetemcomitans*. The lower efficiency of TC as TC released from the microspheres against *A. actinimycetemcomitans* as compared to *P. gingivalis*, could be explained by the higher MIC value (16 times higher than *P. gingivalis*), thus lead to lower bacterial susceptibility to TC. The results show that TC-loaded PHB microspheres has stronger antibacterial effect against *P. gingivalis* than *A. actinomycetemcomitans*, which is in a good agreement with previous studies.[84,85]

CONCLUSIONS

The present study set out to investigate various formulations of biodegradable PHA microspheres for potential treatment of periodontal disease. It was found that the particle size, drug loading, as well as drug release profile could be modified depending on the type of core PHA polymer or M_w of PVA particle stabilizer (i.e., low vs. high M_w) that was used. The %HV content in PHA copolymers had a significant influence on the particle size, drug loading, and encapsulation efficiency. An increase of the %HV content resulted in a decrease in particle size and reduced encapsulation efficiency. Formulations with a higher %HV also demonstrated a higher initial burst release and faster drug release, which was attributed to the crystallinity of the polymer. Microspheres with higher degree of crystallinity (i.e., PHB) exhibited slower drug release rate, attributed to lower drug and water diffusion through the polymer matrix. Data fitted to the Korsmeyer–Peppas model suggested the TC released from PHA microspheres follows a Fickian diffusion mechanism. Furthermore, the drug loading properties and drug release characteristics were found to be influenced by the M_w of PVA used as particle stabilizer. It was observed that low M_w PVA had higher adsorption to PHA particle surfaces and was in general associated with higher drug encapsulation efficiency and lower initial burst release. TC-loaded PHB:low M_w PVA microspheres demonstrated the highest drug loading with a slow release profile sustained over several days, with a high antibacterial activity against periodontitis causing bacteria. This type of microsphere allows prolonged release of antibiotic TC inside the defected periodontal pocket during mechanical cleaning (i.e., scaling and root planning) to kill bacteria for better control of infections. Thus, this PHB microsphere formulation has potential for future application in periodontitis therapy.

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