Published in final edited form as:

Biomaterials. 2012 October ; 33(30): . doi:10.1016/j.biomaterials.2012.06.013.

The microclimate pH in poly(D,L-lactide-co-hydroxymethyl glycolide) microspheres during biodegradation

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Abstract

The microclimate pH (μpH) in biodegradable polymers, such as poly(D,L-lactic-co-glycolic acid) (PLGA) 50/50, commonly falls to deleterious acidic levels during biodegradation, resulting in instability of encapsulated acid-labile molecules. The μpH distribution in microspheres of a more hydrophilic polyester, poly(D,L-lactide-co-hydroxymethyl glycolide) (PLHMGA), was measured and compared to that in PLGA 50/50 of similar molecular weight and degradation time scales. pH mapping in the polymers was performed after incubation under physiological conditions by using a previously validated ratiometric confocal laser scanning microscopic (CLSM) method. Confocal μpH maps revealed that PLHMGA microspheres, regardless of copolymer composition, developed a far less acidic μpH during 4 weeks of incubation compared with microspheres from PLGA. A pH-independent fluorescent probe marker of polymer matrix diffusion of μpH -controlling water-soluble acid degradation products, bodipy, was observed by CLSM to diffuse $\sim 3\text{--}7$ fold more rapidly in PLHMGA compared to PLGA microspheres, consistent with much more rapid release of acids observed from the hydrophilic polymer during bioerosion. Hence, PLHMGA microspheres are less susceptible to acidification during degradation as compared to similar PLGA formulations, and therefore, PLHMGA may be more suitable to deliver acid labile molecules such as proteins.

Keywords

microclimate pH; confocal laser scanning microscopy; hydrophilic polyesters; microsphere; pH distribution; poly(lactic-co-glycolic acid)

Introduction

Poly(D,L-lactide-co-glycolide) (PLGA) is a biodegradable aliphatic polyester that has been widely investigated for controlled delivery of peptides, proteins and vaccine antigens [1–6]. PLGA degrades in aqueous medium via hydrolysis of ester bonds connecting the monomer units in the polymer chain and the final degradation products are lactic and glycolic acid [7].

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A major drawback of PLGA systems is the accumulation of acid degradation products inside degrading matrices, which is associated with a drop in microclimate pH (μpH , i.e., the pH in the aqueous pores of the polymer) [8–10] and unwanted instability of acid-labile PLGA-encapsulated species [11, 12]. The use of poorly soluble bases such as magnesium carbonate, magnesium hydroxide and zinc carbonate as well as blending PEG with the PLGA has been investigated to minimize the drop of pH in protein-loaded PLGA microspheres and to enhance protein stability and release [13–17]. Although co-encapsulation of these additives has shown to improve protein stability and release kinetics [18], the release of peptide/protein drugs from PLGA systems is still commonly incomplete and/or difficult to control [19, 20].

Recently, polyesters with functional pendant hydroxyl groups have been developed and showed attractive degradation and release properties for drug delivery purposes [21–25]. Poly(D,L-lactide-co-hydroxymethyl glycolide) (PLHMGA) in particular, showed tailorable degradation kinetics and release of proteins and peptides from PLHMGA microspheres, which was governed by degradation of the microspheres [22, 23, 25], and reduced aggregation of encapsulated lysozyme and formation of less acylated peptide adducts compared to comparable PLGA formulations [22, 25]. The introduction of hydroxyl groups in the backbone of the PLHMGA copolymers makes the polymer more hydrophilic than PLGA and as a consequence, PLHMGA microspheres have a higher water absorbing capacity than their PLGA counterparts. This increase in hydrophilicity might facilitate the more rapid release of the formed acid degrading products into the release medium, which in turn could inhibit the drop of μpH and subsequently improve the stability of encapsulated species. For example, increased μpH was observed inside PLGA/PEG blend microspheres [10], which showed increased water uptake due to the hydrophilicity of PEG, and significantly improved stability of encapsulated ovalbumin and BSA [17, 26].

In order to have a better insight into the μpH distribution of PLHMGA microspheres, confocal laser scanning microscopy (CLSM), as a noninvasive tool capable of providing detailed μpH mapping, was used to monitor μpH changes during degradation of the microspheres. In the present study, the effects of PLHMGA copolymer composition and polymer solution concentration used to prepare the microspheres on μpH kinetics during polymer bioerosion were studied and compared to that in PLGA 50/50 formulations. The underlying factors accounting for the μpH differences were also explored.

Materials and Methods

Materials

Poly(D,L-lactide-co-hydroxymethyl glycolide)s with copolymer ratios of 65/35 and 75/25 were synthesized and characterized as described before [23, 24]. Poly(D,L-lactide-co-glycolide), end capped, 50/50 with an inherent viscosity (i.v.) of 0.19 dl/g ($M_w=19$ kg/mol) was generously provided by Alkermes Inc. (Cambridge, MA). Polyvinyl alcohol (PVA, MW 9–10 kDa, 80 % hydrolyzed) was from Polysciences (Warrington, PA). The fluorescent probes, Lysosensor yellow/blue[®] dextran (MW=10,000 kDa) and BODIPY[®], FL (MW 292.1) was purchased from Invitrogen (Eugene, OR). Unless otherwise stated, all chemicals were analytical grade or higher and used as received.

Preparation of microspheres

Lysosensor yellow/blue[®] dextran as an acidic pH-sensitive fluorescent probe was encapsulated in PLHMGA and PLGA microspheres by a double emulsion evaporation technique, as described previously [22]. Briefly, 125 μl of dye solution (12 mg/ml) was added to a polymer solution with 350 mg of copolymers/PLGA in 500 μl methylene chloride

(35 % w/w). The mixture was homogenized with Tempest IQ² homogenizer (The VirTis Co., Gardiner, NY) at 20,000 rpm for 30 s to form the w/o emulsion. Next, 500 µl of an aqueous PVA solution (1 % w/w) was slowly added to the first emulsion and a w/o/w was formed by homogenizing the mixture at 20,000 rpm for 30s. The prepared w/o/w was slowly transferred into 5 ml of an aqueous PVA solution (0.5 % w/w) and stirred at room temperature for 2 h to extract and evaporate methylene chloride. The formed microspheres were sieved for 20–45 µm size (USA standard test sieve, sieve No.325 and 635, Newark Wire Cloth Co, Newark, NJ) and washed three times with 100 ml double distilled water and thereafter freeze dried on a FreeZone 2.5 Liter Benchtop freeze dry system (Labconco, Kansas City, MO). Microspheres of copolymer PLHMGA 75/25 from three different polymer concentrations (25, 30 and 35 % w/w) were also prepared followed the same procedure.

Scanning electron microscopy (SEM)

The morphology of the microspheres was studied using a Hitachi S3200 scanning electron microscope (SEM, Hitachi Ltd., Tokyo, Japan). Approximately 1–2 mg of lyophilized microspheres was evenly sprinkled onto a brass stub with double-adhesive conductive tape. Samples were sputter coated with gold under vacuum using DESK II sputter coater (Denton Vacuum LLC, Moorestown, NJ). The images of microspheres were taken at an excitation voltage of 15.0 kV.

Confocal laser scanning microscopy for microspheres imaging

A ratiometric method was employed as essentially described by Ding et al. [10] to map microclimate pH distribution inside microspheres using Zeiss LSM 510-META confocal laser scanning microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). This instrument was equipped with four laser systems and a Zeiss Axiovert 100M inverted microscope. Lysosensor yellow/blue[®] dextran was excited at 364 nm by an Enterprise UV laser and two filters (450 nm and 520 nm) were used to build images. For assessing bodipy diffusion in the microspheres, bodipy was excited at 488 nm by an Argon laser and LP 505 filter was used to construct images, as described by Kang *et al.* [27]. Other instrumental parameters were set up as stated elsewhere [10, 27]. All measurements were conducted using a C-Apochromat 63X water immersion objectives lens with numerical aperture of 1.2.

Standard curve of fluorescent intensity ratio vs. pH

Buffers of pH from 2.8 to 5.8 were prepared using combined 0.1 M citric acid solutions and 0.2 M Na₂HPO₄ solutions. Lysosensor yellow/blue[®] dextran was dissolved in buffer solutions with concentration of 0.8, 1.2, and 2.0 mg/ml. The standard dye solutions were scanned by CLSM. The acquired confocal images were first processed by frame averaging, followed by neighborhood averaging, and applying a median filter as described by Li et al. [28] using Image J software (developed by National Institutes of Health and available on the internet at <http://rsbweb.nih.gov/ij/>) to eliminate signal noise. The standard curve was established by plotting the ratio of mean fluorescent intensities of the dye solutions under two emission wavelengths, 450 nm and 520 nm, versus pH of that solution.

Microclimate pH distribution kinetics inside microspheres

Roughly 15 mg microspheres were suspended into 1 ml phosphate buffer saline (7.74 mM Na₂HPO₄, 2.26 mM NaH₂PO₄, 137 mM NaCl and 3 mM KCl) containing 0.02 % Tween 80 (pH 7.4) (PBST) and incubated in a Glas-Col[®] vial rotator (Glas-Col LLC, Terre Haute, IN) at 40 rpm at 37 °C. At predetermined time points, a small amount of microspheres was separated for confocal imaging study. The release media was also removed for pH measurement using a Corning 430 pH meter (Corning, NY), followed by replacing with

fresh media. The ratio of fluorescent intensities of each pixel having intensity above the threshold value (indicating the fluorescence from release media) at two emission wavelengths (450 nm and 520 nm) was then calculated and related to a pH from the standard curve. In the processed images, each pixel was converted to a color corresponding to pH. The probability of specific pH value inside microspheres was obtained by dividing the amount of pixels corresponding to a specific pH to the total pixels in the images. Pixel ratios that exceeded the limit of standard curve range referred to a pH of either above 5.8 or below 2.8. In such cases, the percentages were plotted as the boundaries of the μ pH distribution curves.

Quantification of water-soluble acids inside PLHMGA and PLGA

Microspheres (80–90 mg) were incubated in PBST buffer under mild agitation at 320 rpm by a KS 130 basic shaker (IKA® Works Inc., Wilmington, NC) at 37 °C for pre-determined times. After incubation, the microspheres were separated from PBST by a brief centrifugation, followed by washing with double distilled water three times. Then, the microspheres were freeze-dried.

80 mg of PLHMGA and PLGA copolymers or dried microspheres were dissolved in 0.5 ml chloroform before adding 3 ml of double distilled water. After a mild vortex mixing, the biphasic solution was left for 10 min and then was centrifuged at 4°C at 4,000 rpm for 5 min. The upper water layer was then quickly removed. The extraction was repeated for 4 times, and finally the water phases were combined.

The water phase was then titrated with 0.1 M NaOH solution to determine the amount of total water-soluble acids. The electromotive force (EMF) was recorded as a function of the moles of titrant added using a pH meter. The quantity of acid was determined by the total added titrant at the end point, which corresponds to the inflection point of the first derivative of potentiometric titration curve. All measurements were performed in triplicate (n=3).

Determination of bodipy diffusivity in PLHMGA and PLGA microspheres

About 1 mg of PLHMGA and PLGA microspheres were suspended in 1 ml of PBST and incubated at 37°C under mild agitation for pre-determined times. After incubation, the microspheres were separated from PBST by a brief centrifugation, followed by adding 1 ml of bodipy in PBST (5 μ g/ml), which was pre-incubated at 37°C. After incubating the mixture at 37°C for 3 hours, a small amount of microspheres was separated for CLSM observation.

Monitoring bodipy uptake in PLHMGA and PLGA microspheres by CLSM and image and data analysis were carried out following procedures reported by Kang et al. [27]. Briefly, the acquired images were analyzed using Image J software to extract intensity profiles along the diameter. The pixel intensity (I)-position (r) data pairs were then normalized by the surface intensity (I_0) and radius (a) of the microsphere, respectively. Because of the linear relationship between fluorescence intensity and probe concentration, the normalized intensity (I/I_0)-position (r/a) data pairs were then fit to the following solution to Fick's second law of diffusion to obtain the effective diffusion coefficient (D) of bodipy in the polymer matrix:

$$\frac{C}{C_0} = \frac{1}{r/a} \sum_{n=0}^{\infty} \left(\operatorname{erfc} \frac{(2n+1) - r/a}{2\sqrt{Dt/a^2}} - \operatorname{erfc} \frac{(2n+1) + r/a}{2\sqrt{Dt/a^2}} \right) \quad (1)$$

where t is the diffusion time. The fitting was done according to a least-squares nonlinear regression using $n=12$ by DataFit software (Oakdale Engineering, Oakdale, PA). All measurements were performed in eight replicates ($n=8$).

Results

Characteristics of PLHMGA copolymers

In Table 1, the characteristics of the protected poly(D,L-lactic acid-*ran*-benzyloxymethyl glycolic acid) (PLBMGA) and deprotected PLHMGA are displayed. The molecular weight and thermal behavior of the copolymers were measured and the characteristics are comparable with those reported in previous studies [22, 23, 29].

Preparation of microspheres loaded with an acidic pH sensitive probe

Microspheres were prepared from PLHMGA with different copolymer compositions (65/35 and 75/25) and PLGA 50/50 using a w/o/w double emulsion-solvent evaporation method. Additionally, for PLHMGA 75/25, solutions with different polymer concentrations were employed to prepare microspheres. Since the development of microclimate pH depends on the size of microspheres [10], the microspheres used for confocal microscopy imaging were sieved to yield particles with a similarly narrow size distribution of 20–45 μm . As can be seen from the scanning electron micrographs (Fig. 1), all microspheres displayed spherical shape and a non-porous surface.

Microclimate pH distribution inside degrading PLHMGA and PLGA microspheres

The microclimate pH distribution inside degrading PLHMGA and PLGA microspheres over incubation under physiologically conditions for one-month was monitored using a CLSM imaging technique. The encapsulated fluorescent dextranconjugated probe, Lysosensor yellow/blue[®] dextran, is sensitive to pH change from 2.8 to 5.8, and this dextran conjugate dye partitions into aqueous pores in polymer similar to encapsulated proteins [8, 10]. A standard curve of the dye correlating its fluorescence intensity ratio at wavelength of 450 nm and 520 nm and pH from 2.8 to 5.8 was established and fitted to a third order polynomial function ($r^2=0.999$) (Fig. 2). This figure shows that the pH sensitivity of the dye is concentration independent as previously reported [10], which ensures that the standard curve is not affected even though the dye concentration changes during incubation. Some important instrument parameters (e.g., detection gain, pinhole, laser power) were adjusted so that within the concentration range from 0.8 mg/ml to 2.0 mg/ml, the images of dye solutions gave fluorescence intensity from 10 to 255 (units of the instrument). In the microspheres images, any value below 10 was regarded as background and the value exceeding 255 was considered saturated. Intensities within the range of 10 to 255 indicated the existence of entrapped dye. The blank regions in the processed images suggested either dye-free pores or a pure polymer phase.

Effect of polymer composition on μpH distribution kinetics— μpH changes were compared in degrading microspheres prepared from PLHMGA of different compositions (65/35 and 75/25) and PLGA 50/50 during incubation in PBST at 37 °C for four weeks, as shown in processed confocal images (Fig. 3) and μpH distribution curves (Fig. 4). Within four weeks incubation of microspheres prepared from PLHMGA 65/35, more than 95 % of pixels in the images gave a fluorescence ratio corresponding to pH out of detection range ($\text{pH}>5.8$), indicating these microspheres did not develop any detectable acidity during that time (Fig. 4A). For PLHMGA 75/25 microspheres, acidity in most aqueous pores with an average pH of 4.8 was observed after one-day incubation, indicative of some acidic impurities in the polymer, although the acidity decreased rapidly with increasing incubation time and almost disappeared by 14 days incubation (Fig. 4B). By contrast, PLGA

microspheres developed μpH as low as 4 during 28 days of study and maintained at an acidic μpH as the degradation of the microspheres continued. The μpH was most acidic after one day of incubation, with around 55 % of pixel domains giving a pH below 5.8. The μpH rose until 2 weeks of incubation before decreasing again. Also note that microspheres made from PLHMGA were observed to be larger than PLGA ones especially at the later stage of incubation, indicative of the higher water-absorbing capacity of PLHMGA.

Effect of polymer concentration used for preparation of microspheres on μpH distribution kinetics

—To investigate the effect of polymer concentration used during microsphere preparation on μpH distribution kinetics, microspheres were prepared using methylene chloride solutions of PLHMGA 75/25 of three different polymer concentrations (25, 30 and 35 % w/w). As shown in the processed confocal images (Fig. 5) and μpH distribution curves (Fig. 6), increasing the polymer concentration decreased the initial μpH after one day of incubation. As the incubation continued, the acidity inside of the microspheres decreased and disappeared completely after 2 weeks. The μpH is typically found to be more acidic in the center of microspheres than the peripheral regions, due to the relatively shorter diffusion length of formed acid degradation products in polymer regions near the microsphere surface.

Quantification of water-soluble acids in PLHMGA and PLGA

The water-soluble acids were extracted from PLHMGA and PLGA raw polymers as well as microspheres and quantified, as the total concentration of these species in the polymer pores is predictive of μpH [30]. The water-soluble acids existing in the raw polymer of PLHMGA and PLGA are attributed to the acid impurities from synthesis and storage, whose quantities were comparable among three polymers (Fig. 7A). The amount of acids in microspheres, on the other hand, were much less than in the raw polymers, consistent with anticipated diffusion out of acids into the outer water phase during the inliquid hardening of microspheres preparation process. However, the quantity of acids extracted from PLHMGA microspheres after incubation in PBST at 37°C was too negligible to be accurately determined, in contrast to PLGA showing the presence of water-soluble acids throughout the incubation period (Fig. 7A). Since this study was conducted using a different batch of polymer from μpH mapping, it did not show detectable acids in PLHMGA 75/25 microspheres during initial incubation, as expected from the mild acidity recorded by CLSM after 1 day of incubation in Fig. 4B. Nevertheless, it is consistent with the μpH measured using CLSM, which demonstrated non-detectable acidity ($\text{pH} > 5.8$) in PLHMGA 65/35 microspheres but an acidic environment in PLGA counterparts during one-month incubation.

pH kinetics in the release media

The pH in the erosion media of microspheres made from different polymers was monitored at the same time point of each μpH mapping, with the buffer being changed weekly. Generally, the pH maintained relatively constant for the release medium of PLGA microspheres. By comparison, the pH declined with the progression of incubation for PLHMGA polymers, with pH of the release medium containing PLHMGA 65/35 lower than that of PLHMGA 75/25 (Fig. 7B).

Determination of diffusion coefficient of bodipy in PLHMGA and PLGA microspheres

In order to test the hypothesis that acid diffusion out of the polymer was responsible for higher μpH in PLHMGA polymer compared to PLGA, the diffusivity of a small hydrophobic fluorescent probe, bodipy inside PLHMGA and PLGA polymer microspheres was determined using CLSM as previously reported [27]. The probe is similar in molecular

weight and polymer/water partition coefficient to the dimer of lactic acid [27, 30]. As a result, its diffusion behavior is expected to be a relative indicator of the diffusion of the acid degradation products. The confocal micrographs of bodipy uptake in different polymer microspheres are displayed in Fig. 8. The dark regions signified the aqueous pores in the microspheres, as the probe concentration in the polymer phase is much higher [27]. The bodipy concentration gradient inside individual microspheres was accurately fit by the solution to Fick's second law of diffusion. (R^2 invariably >0.90 , see Supporting Information Fig. S1). The determined diffusion coefficients (D) of bodipy in degraded microspheres are summarized in Table 2. The diffusivity of bodipy for each polymer maintained relatively constant during the early state of microsphere incubation (<3 days). After one week of degradation, the PLHMGA microspheres became porous and the consequent heterogeneous probe distribution caused a poor fit of the confocal images to the solution of the diffusion equation. The diffusion coefficient of bodipy was highest in PLHMGA microspheres made from PLHMGA 65/35, followed by PLHMGA 75/25 microspheres, and lowest in PLGA microspheres.

Discussion

The acidic microenvironment is often regarded as one of most deleterious factors responsible protein instability inside PLGA delivery systems, particularly rapidly degrading PLGA 50/50. The μpH depends on the concentration of water-soluble acids in the aqueous cavities of polymer matrix. According to the equilibrium model for prediction of μpH developed by Ding et al. [30], a number of factors may contribute to the development of μpH , including: the amount of acidic impurities, the production rate of water-soluble acids, the liberation rate of water-soluble acids out of polymer, the partition coefficient of acids between polymer and aqueous phases, and the acids' dissociation constant (pK_a).

In the present study, we observed that PLHMGA microspheres developed a lower acidic microclimate than that of PLGA 50/50 during one-month incubation under physiological conditions as monitored by CLSM. Since the pK_a of monomer acids of PLHMGA and PLGA are very close, (3.86, 3.82 and 3.53 for lactic acid, glycolic acid and hydroxymethyl glycolic acid, respectively), the pK_a contribution to the μpH differences is negligible. As compared to PLGA, PLHMGAs are more hydrophilic due to the pendant hydroxyl groups on polymer backbone. Because of this more hydrophilic characteristic, monomer acids likely partition more favorably in the water phase of PLHMGA as compared to PLGA. Moreover, since the degradation times of this hydrophilic polyester were shorter than those of PLGA [29], the production of watersoluble acids in PLHMGA is expected to be faster. Therefore, we hypothesized that the μpH -determining water-soluble acid degradation products should be released faster from the more hydrated PLHMGA microspheres in order to counteract the above-mentioned unfavorable factors causing higher acidity in the polymer pores. One important piece of evidence was the lower pH observed in the release media of PLHMGA microspheres (Fig. 7B). Since those acids were quickly released, they did not accumulate in the polymer, leading to an overall less acidic microenvironment in PLHMGAs than in PLGA.

To test our hypothesis, we examined and compared the diffusivity of a small hydrophobic fluorescent probe, bodipy inside PLHMGA and PLGA polymer matrix at the early period of polymer incubation. Bodipy is a good candidate to investigate the transport behavior of small molecules, because i) it is pH-insensitive over pH range of 2 to 10; ii) its transport through PLGA is typically limited by transport through the polymer phase; and iii) it displayed a linearity of emission intensity-concentration relationship under our experimental conditions [27]. The effective diffusion coefficient (D) of bodipy in the polymer matrix was determined after incubating degraded microspheres in bodipy solution for 3 hours and then

fitting the concentration gradients inside individual microspheres to the solution of Fick's second law of diffusion. This method was confirmed by the fact that i) the bodipy uptake time did not change the value of D (see Supporting Information Table S1), which is consistent with our previous data [27] and ii) the excellent fit of experimental data to equation (1). Since the liberation of water-soluble acids was controlled mainly by polymer diffusion at initial stage of polymer erosion when the pore connectivity in the polymer matrix was still low [27], the higher mobility of bodipy in PLHMGA should indicate a similar trend in the transport behavior of water-soluble acids (Table 2).

The high permeability of PLHMGA to water-soluble acids relative to PLGA could be explained as follows. First, due to the introduction of pendant hydroxyl group on PLHMGA's backbone, an elevated amount of water is expected to associate with the polymer phase upon microsphere incubation, which could act as a strong plasticizer [31], leading to the relaxation of polymer chains, thereby increasing the diffusivity of degraded acids through polymer phase. Moreover, the more porous internal structure in PLHMGA microspheres appearing with the progression of incubation, suggested by more isolated dark spots in Fig. 8, caused a higher effective diffusivity of acidic degradation products through the polymer matrix, as the diffusion across the aqueous pores is several orders of magnitude higher than diffusion in the polymer phase.

Increasing the ratio of hydroxymethyl glycolic acid from 25 % to 35 % increases the hydrophilicity of PLHMGA. Despite the hydrolysis rate of the polymer is raised [23], the diffusion of acid degradation products out of the polymer was further facilitated, causing an even more neutral μpH inside PLHMGA 65/35 microspheres than in PLHMGA 75/25. This was supported by the higher diffusion coefficient of bodipy measured in PLHMGA 65/35 microspheres than in that of PLHMGA 75/25 (Table 2) and the more acidic pH in the corresponding release media (Fig. 7B).

The mild acidity in PLHMGA 75/25 microspheres recorded after one-day incubation described in Fig. 4 and Fig. 6 was likely due to the acid impurities existing in the polymer following polymer synthesis, purification and storage. However, as the incubation proceeds, those acids were gradually released, giving rise to a μpH increase and a neutral microenvironment after 2 weeks. Using polymer from a different batch, acidity was not observed during the entire course of incubation, (see Support Information Fig. S2), suggesting the role of acid impurities playing in initial low μpH . Increasing the polymer concentration in methylene chloride when fabricating microspheres decreased the initial μpH after one-day incubation. This can be rationalized by the fact that microspheres made from solutions with higher polymer concentration possessed more acidic impurities. Additionally, such microspheres usually have denser structures [32], which impede the liberation of these water-soluble acids at initial incubation. However, because of the higher permeability of PLHMGA copolymer, the effect of polymer concentration had no significant influence on μpH kinetics as the incubation continued. Therefore, the μpH inside of the microspheres all increased to neutral range (above 5.8) consistent with the release of acid impurities as well as degradation products into the incubation medium.

Conclusion

The microclimate pH (μpH) inside degrading microspheres prepared from a novel hydroxylated aliphatic polyester; poly(lactide-co-hydroxymethyl glycolide) (PLHMGA) was quantitatively mapped by confocal laser scanning microscopy during incubation under physiological conditions. Consistent with previous data of improved stability of PLHMGA encapsulated proteins/peptides, we observed a reduced μpH in PLHMGA microspheres made from copolymer 65/35 and 75/25 during one-month incubation relative to comparable

PLGA formulations. The μpH inside PLHMGA microspheres made from copolymer 75/25 during the first two weeks incubation decreased with increasing the polymer concentration. By comparing the pH of release media of PLHMGA and PLGA microspheres and the effective diffusivity of a small fluorescent probe, the data strongly suggests the faster liberation of water-soluble acids in PLHMGA was responsible for its more neutral microenvironment. This study shows that PLHMGA microspheres are potential carriers for controlled delivery of acid-labile biomacromolecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Sima Rahimian in Utrecht University for her help in synthesis of PLHMGA copolymers. This work was supported by NIH R01 HL 68345 and the University of Michigan Gordon Amidon Fellowship to Yajun Liu.

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Fig. 1. Scanning electron micrographs of microspheres prepared from PLHMGA 75/25 with 25 % w/w (A), 30 % w/w (B), 35 % w/w (C) polymer solution concentration, and PLHMGA 65/35 (D) and PLGA 50/50 (E) prepared from a 35 % w/w solution concentration.

Fig. 2.

The pH sensitivity of confocal measurement of Lysosensor yellow/blue® dextran at concentration of 2 mg/ml (), 1.2 mg/ml () and 0.8 mg/ml (). The third-order polynomial curve fitting the data was $Y = -0.0582x^3 + 0.7221x^2 - 2.5676x + 3.0213$, where $Y = I_{450nm}/I_{520nm}$ and $x = \text{pH}$, $r^2 = 0.999$.

Fig. 3. Processed confocal images of (A) PLHMGA 65/35, (B) PLHMGA 75/25 and (C) PLGA 50/50 microspheres during incubation in PBST at 37 °C for 4 weeks. Images were taken at 1 (A₁-C₁), 7 (A₂-C₂), 14 (A₃-C₃), 21 (A₄-C₄) and 28 (A₅-C₅) days.

Fig. 4.

The μ pH distribution kinetics of microsphere formulations during incubation at 37°C in PBST for 1 day (○), 7 days (□), 14 days (△), 21 days (◇) and 28 days (▽). Microspheres were prepared from (A) PLHMGA 65/35, (B) PLHMGA 75/25 and (C) PLGA 50/50, and sieved to 20–45 μ m size for the confocal pH mapping study.

Fig. 5. Processed confocal images of PLHMGA 75/25 microspheres made from (A) 25% w/w (B) 30% w/w (C) 35% w/w of polymer concentration during incubation in PBST at 37 °C for 4 weeks. Images were taken at 1 (A₁-C₁), 7 (A₂-C₂), 14 (A₃-C₃), 21 (A₄-C₄), 28 (A₅-C₅) days.

Fig. 6.

The μ pH distribution kinetics of microsphere formulations during incubation at 37°C in PBST for 1 day (), 7 days (), 14 days (), 21 days (), and 28 days (). Microspheres were prepared from PLHMGA 75/25 of (A) 25 % w/w (B) 30 % w/w and (C) 35 % w/w of polymer concentration, and sieved to 20–45 μ m size for the confocal pH mapping study.

Fig. 7.

Comparison of PLHMGA and PLGA kinetics of total extracted water-soluble acid by titration (A) and pH in the erosion medium (B) recorded for PLHMGA 65/35 (), PLHMGA 75/25 () and PLGA 50/50 () microspheres during incubation in PBST at 37 °C for 4 weeks. The buffer was changed weekly for both experiments and the pH was measured before each buffer change. Symbols represent mean $\pm$ SD (n=3). * acid content in PLHMGAs was below the limit of detection ($<0.002 \mu\text{mol/mg}$) (Figure 7A) throughout the incubation period.

Fig. 8. Representative CLSM micrographs of the 3-h developed fluorescent intensity gradients of bodipy in A) PLHMGA 6535 B) PLHMGA 7525 and C) PLGA5050 microspheres, which had undergone 0 (A₁-C₁), 1 (A₂-C₂), 3 (A₃-C₃) and 7 (A₄-C₄) days of degradation under physiological conditions. The scale bar represents 20 μ m.

Table 1

Characteristics of PLBMGA and PLHMGA copolymers

polymer	feed ratio D,L ^a /M ^b	copolymer composition (NMR)	M _n (kg/mol)	M _w (kg/mol)	T _g (°C)
PLBMGA	75/25	78/22	16	35	36
	65/35	70/30	24	51	41
PLHMGA	75/25	80/20	13	30	49
	65/35	69/31	22	45	47

^aD,L = D,L-lactide

^bM = BMIMG (benzyloxymethyl methyl glycolide)

Table 2

Diffusion coefficient of bodipy in degraded microspheres after 3 hours incubation in bodipy solution

Pre-incubation time (day)	PLHMGA65/35 D($\times 10^{-12}$ cm ² /s)	PLHMGA75/25 D($\times 10^{-12}$ cm ² /s)	PLGA50/50 D($\times 10^{-12}$ cm ² /s)
0	2.6 $\pm$ 0.6 ^a	1.6 $\pm$ 0.6	0.40 $\pm$ 0.05
1	2.8 $\pm$ 0.4	1.6 $\pm$ 0.3	0.5 $\pm$ 0.1
3	2.6 $\pm$ 0.8	1.6 $\pm$ 0.4	0.46 $\pm$ 0.12
7	-- ^b	-- ^b	0.5 $\pm$ 0.10

^a values represent mean $\pm$ SD, n=8.

^b poor fit of D ($R^2 < 0.90$) due to the increased aqueous pores and a consequent heterogeneous probe distribution.