Analysis of Transient Protein Distribution in PLGA Microparticles during Polymer Degradation and in Vitro Release

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Introduction

In the efforts of developing predictable release profiles, such as a sustained release, zero-order release or pulsatile release, the transient protein distribution in the microparticles over long-term release is one of the primary parameters together with polymer degradation and transport characteristics in both bulk erosion and surface erosion (Siepmann *et al*., 2001). Although the significance of accurate analysis of this parameter is evident, it still remains a challenge to quantify transient protein distribution profiles because it is difficult to analyze the protein distribution at original locations without extracting the proteins out of the microparticles.

There are only few reports about protein distribution investigations in the biodegradable microparticles. As some proteins can be fluorescently labeled, confocal laser scanning microscopy (CLSM) was first used to study the detailed protein distribution inside the microspheres (Batycky *et al*., 1997; Rafati *et al.*, 1997; Yang *et al*., 2001). Later, Fourier Transform Infrared Spectroscopy (FTIR) techniques were investigated to visualize lysozyme distribution and conformation in a biodegradable polymer matrix (van de Weert *et al*., 2000). No reports on the quantification of transient protein distribution profiles yet.

As is well-known, transmission electron microscopy (TEM) is a powerful imaging technique for internal structures of materials; however it was not used very often for the characterization of microparticles. This work addresses the use of TEM as a start of quantification of transient OVA distribution in PLGA microparticles fabricated by double-emulsion (w/o/w) method. In order to obtain a more accurate analysis, 2-D TEM images of microparticle sections are used to reconstruct 3-D images of transient protein distribution. Furthermore, the OVA distribution profiles were examined by pixel analysis of the images.

Material and Methods

Poly (lactide-co-glycolide) (PLGA) 75:25 (Resomer 755, Boehinger Ingelheim, Ingelheim, Germany) was obtained. Ovalbumin (OVA) (Grade V, MW = 44K), bicinchoninic acid (BCA) protein assay, and poly (vinyl alcohol) (PVA) (MW 30,000-70,000) were purchased from Sigma (St. Louis, MO, USA). Methylene chloride (MC) was obtained from Fisher Scientific (Pittsburgh, PA, USA). Eponate 12 resin was obtained from Electron Microscopy Sciences (Hatfield, PA, USA).

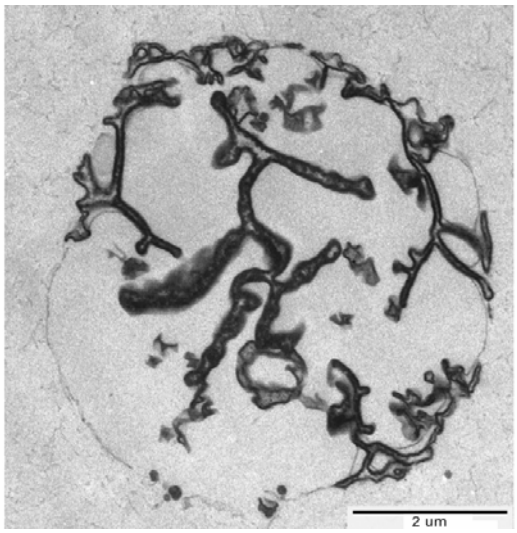
The microparticles were fabricated by double-emulsion method. The first TEM imaging was carried out before incubation (Bozzola *et al*., 1999). During incubation, the microparticles were sampled again for TEM observation (every 20 days) until incubation went to near the end and little protein could be identified by TEM. In addition, three complete sets of consecutive images of a single OVA-loaded microparticle were obtained before and during degradation (0 day, 30 days and 60 days).

The consecutive TEM images were treated for the equal brightness and contrast by the NIH software Image J. Then montages for a specific particle were generated in Image J. Finally, the 3-D images were reconstructed based on the consecutive images via the software of Amira 3.0 (Template Graphics Software, Inc., San Diego, CA, USA). Pixel analysis was performed for the protein distribution evaluation.

Results and Discussions

The Protein Distribution Profiles in PLGA 75:25 Microparticles

Under TEM examinations, the variation of brightness and contrast reflect the internal characteristics of the slides, which are the cross-sections of microparticles. The polymeric background without OVA present turned out to be uniform and moderate in brightness and contrast. The OVA-distributed areas are characterized by the dark color with a high contrast with the background. The TEM images of protein-loaded microparticles made of PLGA 75:25 demonstrated the vivid protein distribution profiles (Figure 1). The dark colors faded with time, which means the decrease of protein concentration over time.



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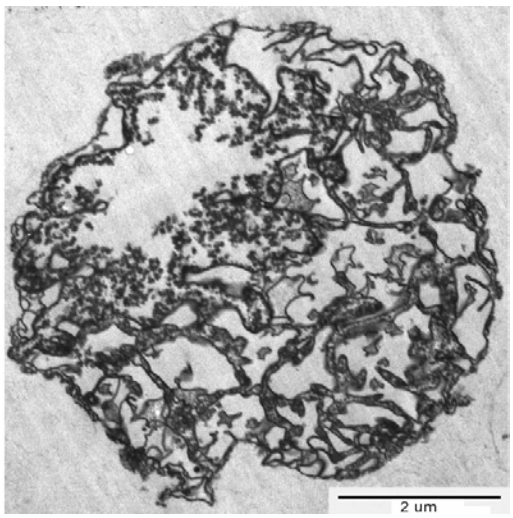

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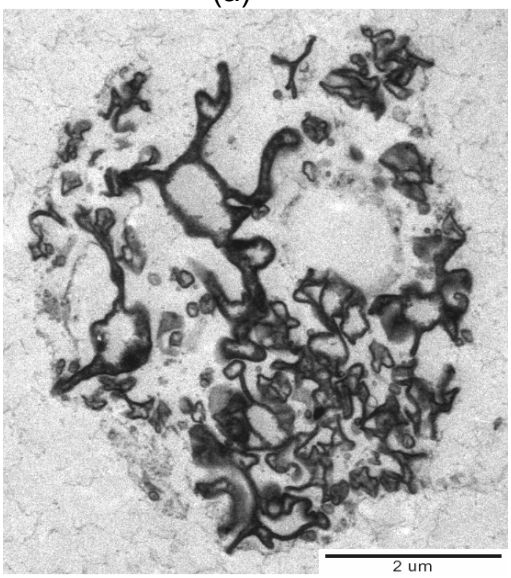

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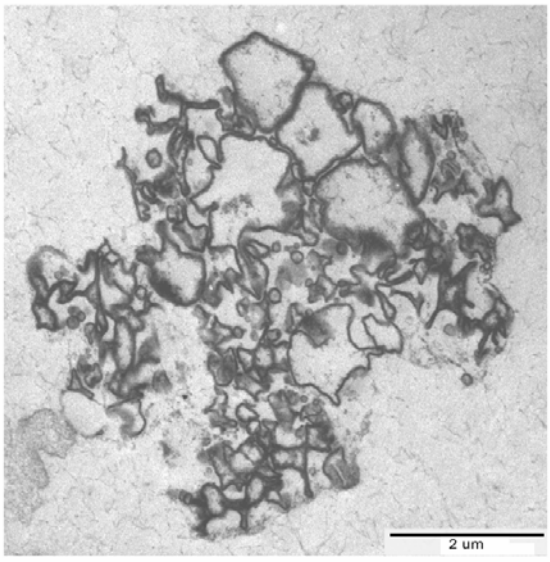

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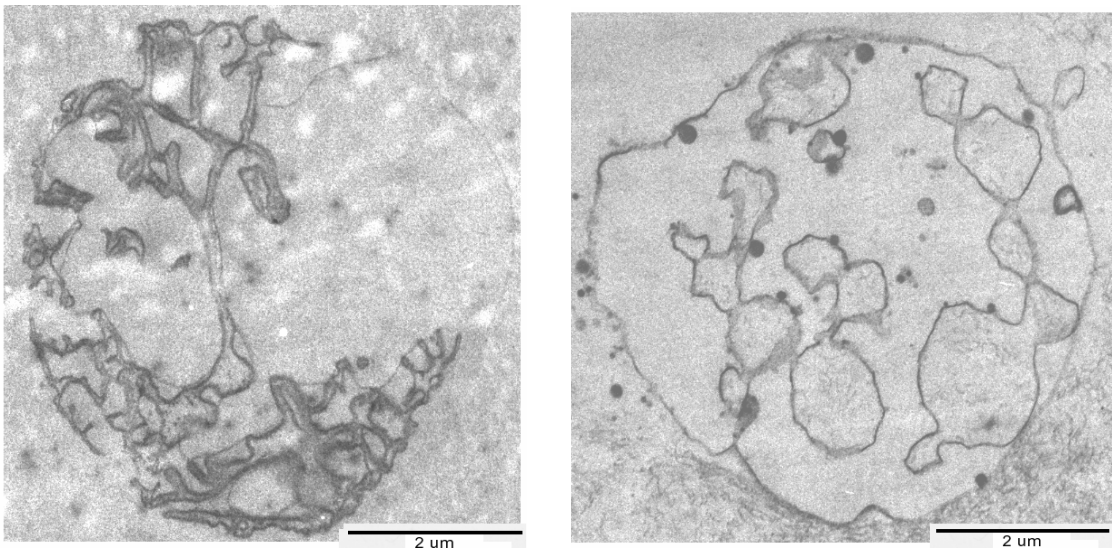
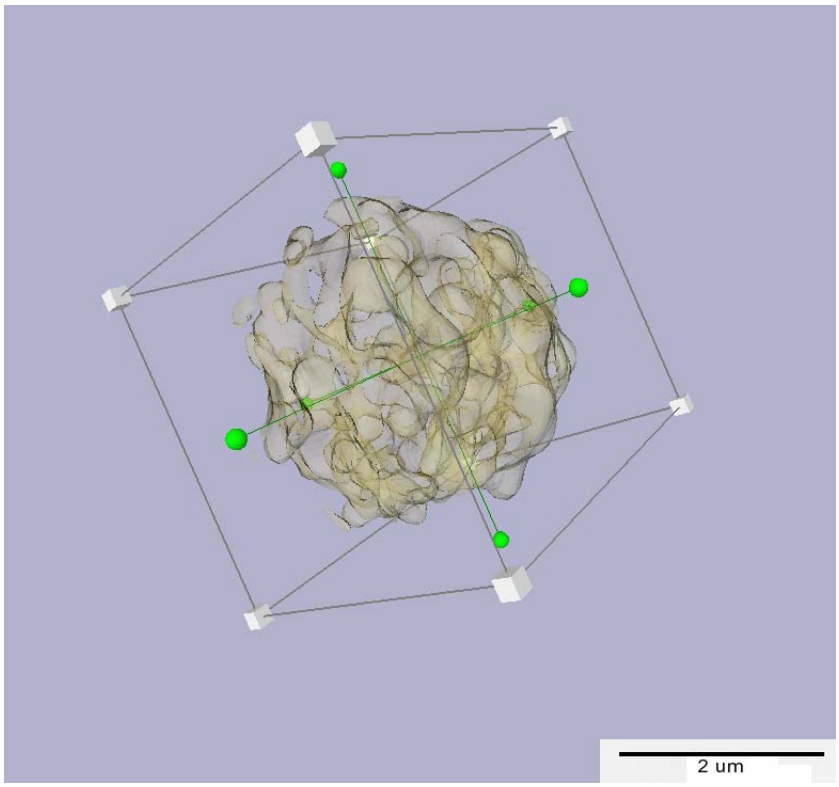


(e) (f)

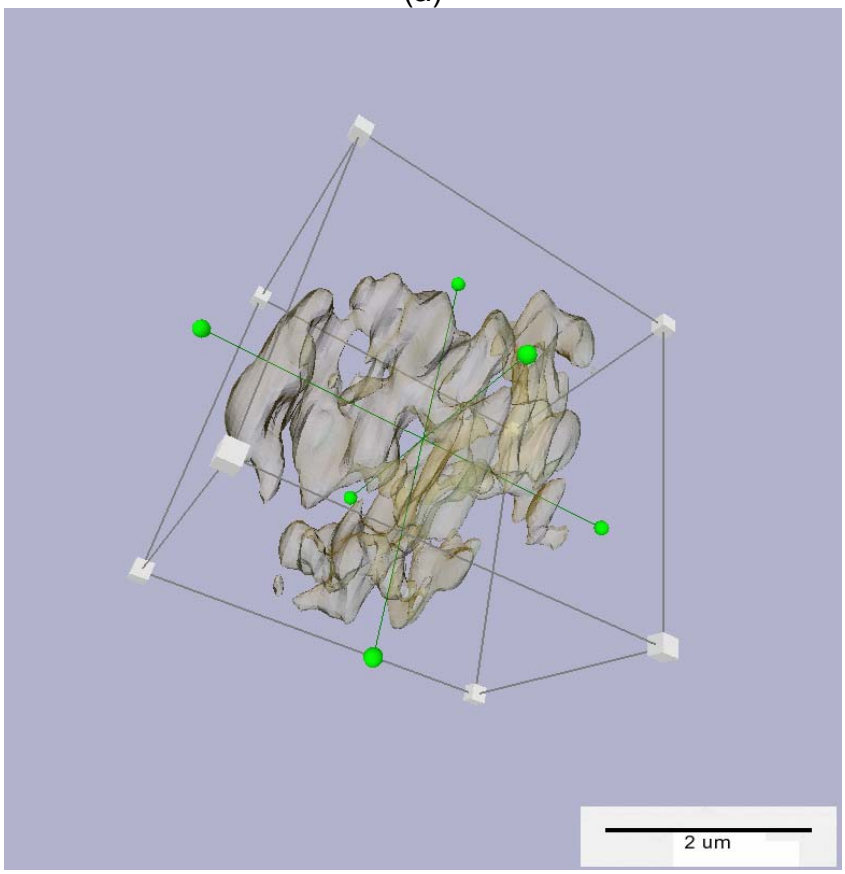
Figure 1 TEM images of OVA-loaded microparticles before and during degradation. Wall material is PLGA 75:25. Fabricated by double-emulsion method. At incubation time of: (a) 0day; (b) 20days; (c) 40 days; (d) 60 days; (e) 80 days; (f) 100 days.

The 3-D Reconstruction of the Protein Distribution Profiles

While a 2-D TEM image can only show the protein distribution in one specific cross-section of a microparticle, a 3-D image of a microparticle reconstructed from the TEM pictures is able to demonstrate the overall protein distribution in the entire particle. Each TEM image obtained from series section represents the specific protein distribution profile at a certain position along the axial direction. Therefore, the protein distribution profile in the entire microparticle was obtained. The protein distribution profiles in the microparticles were visualized by the 3-D reconstruction of the microparticles base on the montages obtained by series section (Figure 2). The yellow color represents protein molecules. The color density in the images, in general, represents protein concentration, which means, the higher color density, the higher protein concentration. Correspondently, the blank areas without any yellow color represent the areas of the microparticle in which no protein distributed. Therefore, the real stereo protein distributions which cannot be determined directly were highlighted by the 3-D reconstructions. Furthermore, the 3D movies demonstrate a more vivid protein distribution observed through every orientation of 360 degree. The 3-D images of the microparticles. It should be emphasized that no polymer molecules are shown the 3-D images and the 3-D images only represent the protein-occupied pore structures of the microparticles, not the original particles. The protein distribution profiles for PLGA 75:25 microparticles over incubation time show a graded concentration decrease from the beginning (0 day) to 60 days. When the incubation proceeded to 60 days, few protein molecules were observed to be left.



(a)



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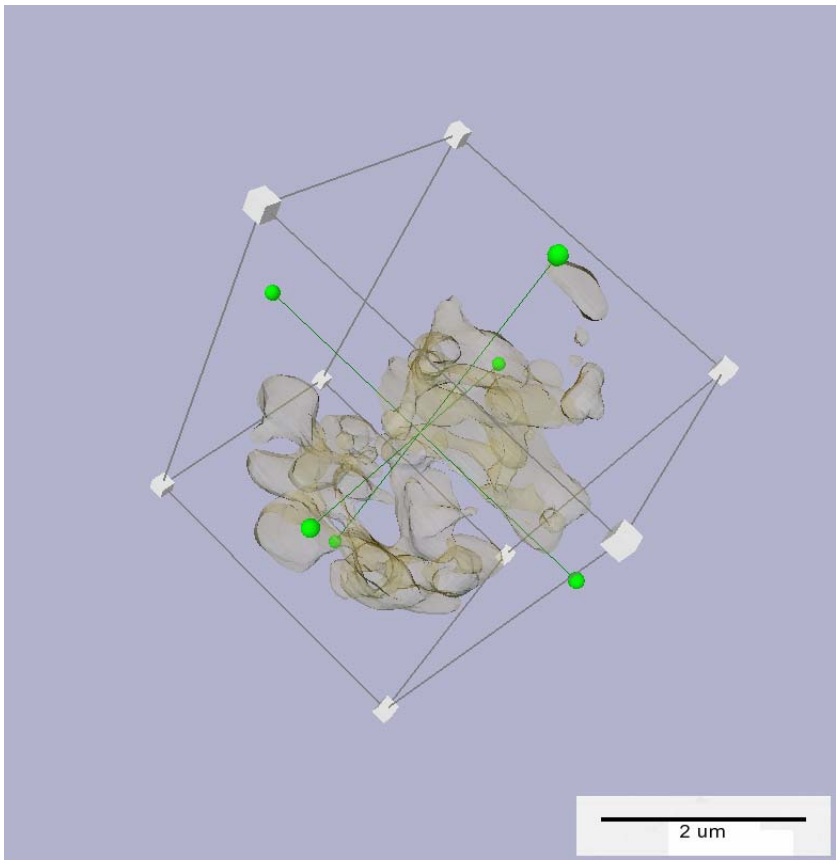
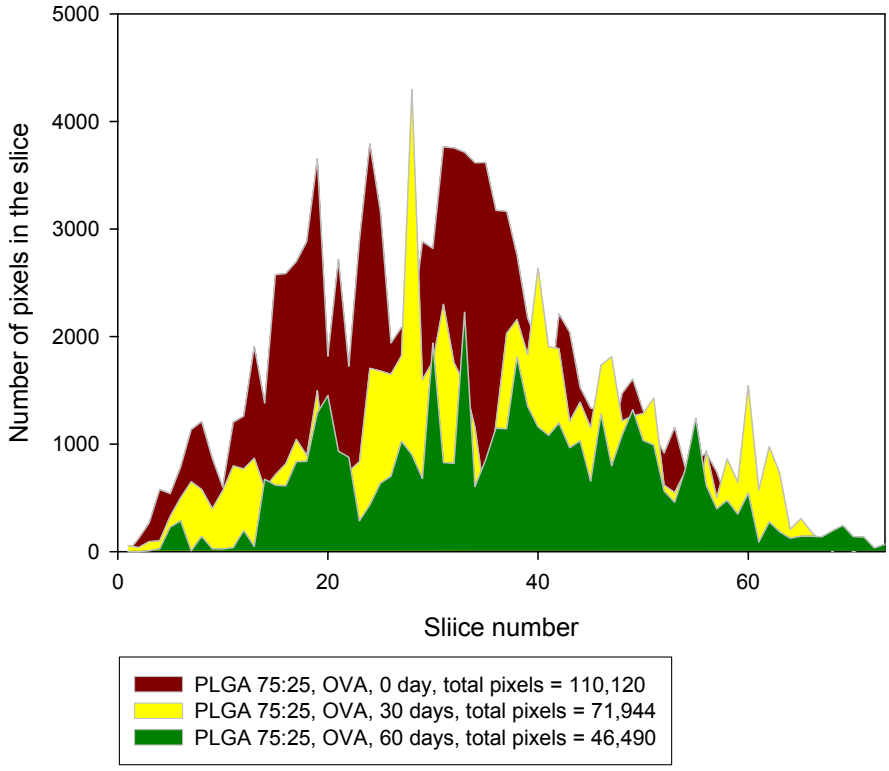


(c)

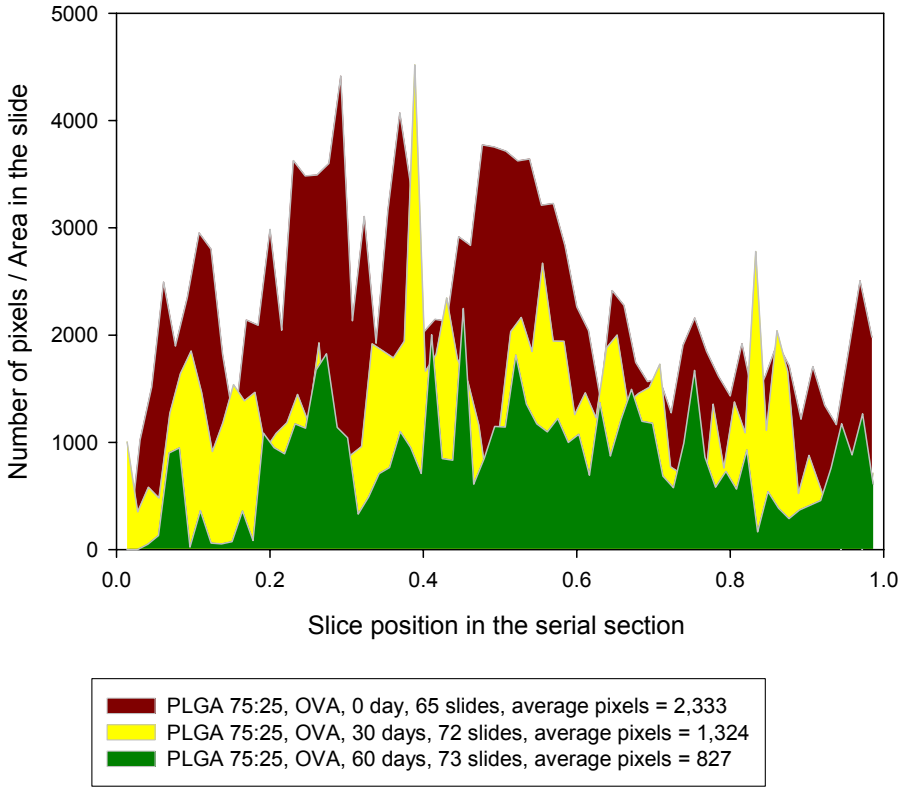
Figure 2 3-D images of the transient protein distribution profiles in OVA-loaded microparticles. Wall material is PLGA 75:25. Fabricated by double-emulsion method. At incubation time of: (a) 0 day; (b) 30 day; (c) 60 days.

Pixel Analysis of the TEM Images

The pixel analysis for the dark areas, which are protein-distributed areas, can be used for this quantification evaluation. The number of pixels in the dark areas reflects the quantity of protein molecules. The total OVA distribution profiles in the particle slides for PLGA 75:25 after series section at a time series of 0 day, 30 days and 60 days are shown in Figure 3.In Figure 3 (a), protein release over time was indicated by the decrease of total pixels from 110K to 72K in the first 30 days, and from 72K to 46K in the second 30 days. Therefore, about 34% of protein was released in the first month and another total release of 24% occurred in the second month. When the degradation and release proceeded to 60 days, a percent of 42% proteins was left in the microparticle. The graded decrease of protein concentration was shown in Figure 3 (b) and the average number of pixels drops from 2.3K to 1.3K in the first month and to 0.8 K in the second month. These evaluations can be used to compare with the OVA release curves directly measured by BCA assay. Also, these data are valuable in studying the transport properties of proteins through pores in the degrading materials.



(a)



(b)

Figure 3 Transient protein distribution analysis of TEM montages in terms of pixels for OVA-loaded microparticles over incubation time. Wall

material is PLGA 75:25. Fabricated by double-emulsion method. Sampled at degradation time of 0 day, 30 days and 60 days respectively. (a) Total protein distribution in particle slices; (b) Protein concentration distribution in the microparticles.

Pixel Analysis Compared to Experimental Protein Release Curves

A comparison of the results from pixel analysis and experiments was listed in Figure 4. Experimentally, a small OVA release (2.7%) associated with the surface proteins was observed for the initial burst followed by the sustained release mainly from internal of the particles over the 60 days. At time point of 30 days, a percentage of 32.3% total OVA was released. By the incubation of 60 days, the percent of cumulative OVA release reached 53.9%. These experimental results show great consistency with the results from pixel analysis of the TEM montages (Approximately 34.7% for the first 30 days and 57.8% for the entire 60 days). Although the latter results were obtained from the analysis of a single microparticle, the protein distribution profile in this microparticle was apparently able to represent the typical protein distribution profiles of all the particles. Therefore, when the model particles are well sampled representatively under TEM investigations, quantification of protein release by pixel analysis is a potential accurate method.

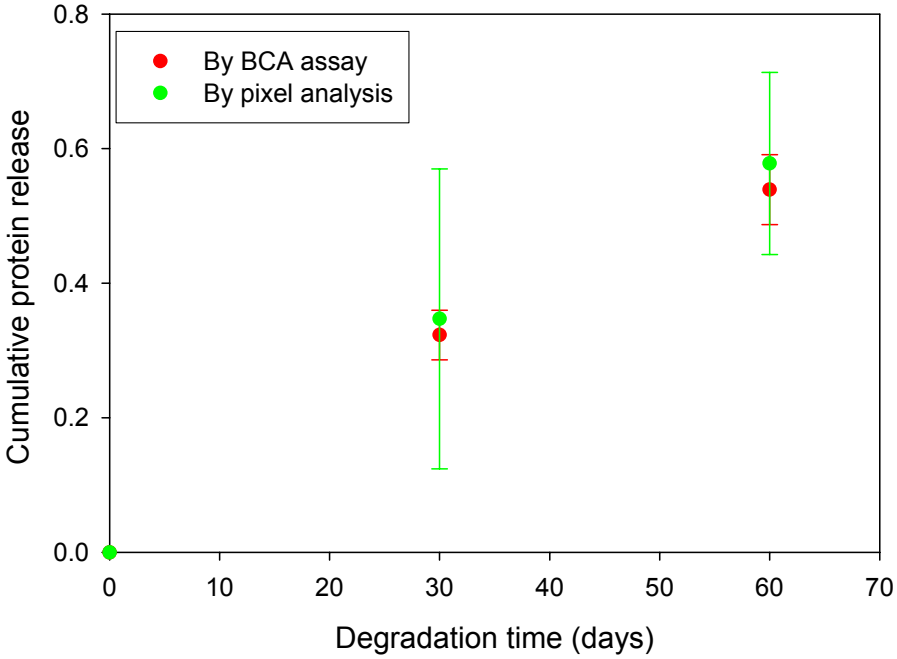


Figure 4 Comparison of the cumulative OVA release from PLGA microparticles obtained from pixel analysis with the experimental results by BCA assay. Wall material is PLGA 75:25. Fabricated by double-emulsion method. Sampled at incubation time of 0 day, 30 days and 60 days respectively.

Conclusion

TEM was used to thoroughly analyze transient protein distribution of PLGA microparticles before and during *in vitro* release experiments. From TEM images, it was found that the protein distribution in a microparticle was not homogeneous but uniformly distributed. The 3-D mages and pixel analysis provided a foundation for a more quantitative

approach to the analysis of transient protein distribution and release of polymeric microparticles fabricated by double-emulsion method.

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