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Using TEM to couple transient protein distribution and release for PLGA microparticles for potential use as vaccine delivery vehicles

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Abstract

In the development of tunable PLGA microparticles as vaccine delivery vehicles, it is important to understand the drug distribution within the microparticle over time as well as the long-term release of the drug during polymer degradation. This study addresses the transient 3-D drug distribution in PLGA microparticles during in vitro degradation. Specifically, poly (lactide-co-glycolide) (PLGA 75:25) microparticles containing ovalbumin (OVA) as a model protein were fabricated by double-emulsion (w/o/w) method. The microparticles were incubated at 37 °C and 250 rpm in PBS buffer (pH 7.4) over a 100-day period. The in vitro polymer erosion, transient protein distribution profiles and protein release behaviors were investigated. Protein release profiles were determined via spectrophotometry using a BCA assay for the solution. Transmission electron microscopy (TEM) images were obtained for the OVA-loaded microparticles before and during degradation (0 day, 30 days and 60 days), and the corresponding 3-D constructions were developed. From the 3-D constructions, the overall protein distribution of the entire microparticle was vividly reflected. Pixel number analysis of the TEM images was used to quantify transient protein distribution. The transient protein release obtained from the TEM analysis was in good agreement with the BCA analysis. This technique provides an additional tool in helping develop polymer matrices for tunable delivery vehicles in vaccination and other drug delivery scenarios.

Keywords: PLGA; Transmission electron microscopy (TEM); Protein distribution; Biodegradable microparticles; Ovalbumin; Polymer erosion; Polymer degradation; Protein release; Transient release; Time-dependent

1. Introduction

Controlled protein release from biodegradable microparticles has been extensively investigated as potential vehicles for vaccine delivery in the past two decades. Whether for sustained release or pulsatile, the transient protein distribution in the microparticles over long-time, together with polymer degradation, transport characteristics in both bulk erosion and surface erosion, are the primary parameters in determining the efficacy of this methodology [1].

In modeling protein distribution, a uniform protein distribution is usually assumed [2–4]. However, protein distribution profiles may vary among the microparticles for different wall polymer or encapsulated protein due to polymer–protein interactions [5–7]. In addition, various microencapsulation processes involve a number of factors which directly influence the protein encapsulation and the subsequent protein distribution profiles in the microparticles [8–11]. Moreover, although the significance of accurate analysis of protein distribution is apparent, it still remains a challenge to quantify transient protein distribution profiles during the degradation process.

There are, however, a few reports investigating protein distribution in biodegradable microparticles. As some proteins can be fluorescently labeled, confocal laser scanning microscopy (CLSM) was first used to study the detailed protein distribution inside microspheres [3,12,13]. Later, Fourier transform infrared spectroscopy (FTIR) techniques were investigated to determine lysozyme distribution and conformation in a biodegradable polymer matrix [14]. Although both CLSM and FTIR are capable of investigating the internal

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properties of the microparticles, their resolutions cannot fulfill the requirement of detailed protein distribution in the cross-sections of the microparticles [15,16].

Although, transmission electron microscopy (TEM) is a powerful imaging technique with high resolution for internal structures of materials, it has not been used as often as SEM for the characterization of microparticles [17]. In the area for controlled drug release, the early use of TEM was directed towards the investigations of the outer layer and inner core of double-walled microparticles [18]. Later, Sandor et al. used TEM for pore size analysis of microparticles [19]. Camli et al. investigated the bulk structure of some macroporous latex particles by TEM [20]. Other research teams employed TEM to confirm the shape and size of the microparticles or nanoparticles compared with the images obtained from SEM [21–23]. However, there have been no reports to date on the quantification of protein distribution profiles coupled with transient release.

Compared with other imaging techniques such as CLSM and FTIR, the TEM methods have both advantages and disadvantages [15–17]. The outstanding advantages are that TEM methods can provide the increased resolution and the visualization of the particle ultrastructures. The signal/noise ratio of the protein resolution in the TEM images (comparison of the highlighted areas with the background) is high enough to be incorporated into the montages of the particles without losing any structure information. The montages are the foundation of the resulting 3-D reconstructions. The disadvantages of TEM methods lie in the relatively time-consuming sectioning and staining work. However, as will be shown, TEM can provide a thorough analysis of the protein distribution profiles in the degrading microparticles.

This work addresses the use of TEM as a method to characterize transient protein distribution during long-term microparticle degradation to couple transient protein distribution with delivery. Here transient degradation of PLGA microparticles fabricated by a double-emulsion (w/o/w) method encapsulating ovalbumin (OVA) is investigated. Three-dimensional constructions of transient protein distribution are developed and image analysis is used to quantify protein distribution and release within the microparticles. Finally, protein release rates are compared to the in vitro cumulative protein release over the transient degradation period.

2. Materials and methods

Poly (lactide-co-glycolide) (PLGA) 75:25 (Resomer RG 755, Boehinger Ingelheim, Ingelheim, Germany) were obtained. The average molecular mass was 68 kDa. Ovalbumin (OVA) (Grade V, 44 kDa), bicinchoninic acid (BCA) protein assay, and poly (vinyl alcohol) (PVA) (30–70 kDa) 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).

2.1. Preparation of microparticles by double-emulsion method

PLGA 75: 25 was used as the microparticle material, MC was used as the organic solvent, PVA was used as the emulsion stabilizer and OVA was the model protein for the degradation and release experiments. Blank microparticles, made by the same procedure, were used as the control.

The microencapsulation procedure was based on the double-emulsion (w/o/w) method. Briefly, 1 g PLGA was dissolved in 25 ml MC; then 1 ml protein solution (30 mg/ml) or DI water was added to 9 ml PLGA solution and sonicated by a sonic dismembrator (Band 1, Model 100, Fisher) for 15–25 s. This w/o emulsion was then poured into 50 ml PVA solutions and sonicated for another 15–25 s with the same sonicator. The double emulsion (w/o/w) was stirred by a Barnant mixer (Band 1, Series 20, IL, USA) for a period of time between 15 and 24 h. The suspension was then centrifuged (Marathon 8 K, centrifuge, Fisher, St. Louis, MO, USA) at 3800 rpm for 15 min. The solids were collected and washed three times with DI water. The microparticles were immersed in liquid nitrogen and then freeze-dried (Freeze Dryer 4.5, Labconco, MO, USA) at −50 °C and 10 μm Hg overnight.

2.2. Determination of protein loading of microparticles

The total protein loading efficiencies of the microparticle were examined by the methods of Coombes et al. [24]. Briefly, an amount of microparticles between 8 and 10 mg was accurately weighed and then redispersed in 3.0 ml of 0.1 M NaOH containing 5% (w/v) SDS. The mixture was incubated overnight in an orbital shaker and then centrifuged. Finally, a BCA protein assay was used to determine the protein concentration in the supernatant. The protein standards were treated in 0.1 M NaOH containing 5% (w/v) SDS. Provided with the measured protein concentration, the total protein loading efficiency (%, w/w) was calculated and expressed as the amount of encapsulated protein relative to the weight of microparticles and as the amount of encapsulated protein relative to the total protein used for microencapsulation. Three samples were assayed for each formulation.

Measurement of surface protein loading was also based on the work of Coombes et al. [24]. Here, 3–4 mg microparticles were accurately weighed and treated with 1 ml of 2% (w/v) SDS solution for 4 h in an orbital shaker at room temperature. Similar to the measurement procedure of total protein loading described above, the samples were centrifuged and the supernatant was analyzed with a BCA assay. The samples were assayed in triplicate. The surface protein loading (%, w/w) was also expressed as the protein percentage in the microparticles and as the protein encapsulated relative to the initial protein weight for microencapsulation.

2.3. Characterization of microparticles by SEM and TEM

The surface properties and size distribution analysis was characterized by scanning electronic microscopy (SEM). The