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Quantitative three-dimensional analysis of poly (lactic-co-glycolic acid) microsphere using hard X-ray nano-tomography revealed correlation between structural parameters and drug burst release

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ABSTRACT

The objective of this study was to investigate the use of transmission hard X-ray nano-computed-tomography (nano-CT) for characterization of the pore structure and drug distribution in poly (lactic-co-glycolic acid) (PLGA) microspheres encapsulating bovine serum albumin and to study the correlation between drug distribution and burst release. The PLGA microspheres were fabricated using a double-emulsion method. The results of pore structure analysis accessed with nano-CT were compared with those acquired by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Surface pore interconnectivity and surface protein interconnectivity were obtained using combined nano-CT and pixel analysis. The correlation between surface protein interconnectivity with the initial burst release across various tested formulations was also analyzed. The size, shape, and distribution of the pores and protein could be clearly observed in the whole microsphere using nano-CT, whereas only the sectional information was observed using SEM or CLSM. Interconnected pores and surface connected pores could be clearly distinguished in nano-CT, which enables the quantitative analysis of surface pore interconnectivity and surface protein interconnectivity. The surface protein interconnectivity in different formulations correlated well with the burst release at 5–10 h. Nano-CT provided a nondestructive, high-resolution, and three-dimensional analysis method to characterize the porous microsphere.

1. Introduction

Biodegradable polyesters such as poly (lactic-co-glycolic acid) (PLGA) have been widely used as carriers of microspheres for controlled release of macromolecular drugs due to their biocompatibility [1–3]. By releasing drug molecules in a controlled manner over extended periods of time from a single administration, sustained-release systems have the potential to maintain drug concentrations, diminish adverse effects caused by extremes in concentration and repeated administrations, and improve patient compliance as compared with conventional regimens.

It is well known that the release profiles of microspheres are determined by various factors including the physicochemical properties of the drug and polymer, the pore structure of the matrix system (pore size, porosity, connectivity, and tortuosity), drug distribution, polymer dissolution, degradation, and erosion [4]. Initial burst of microspheres upon immersion in a release medium either *in vitro* or *in vivo* is a distinct drawback in terms of an efficient and desirable release profile. The immediate drug release might occur due to the protein that is either associated with the surface of the microspheres or located in the channels and pores connected to the surface, which was easily accessible once the particle was hydrated [5–7]. The presence of the pores does not only increase the mobility of the involved species (drug molecules, acids, and bases), but also fundamentally alters the underlying drug release mechanisms of PLGA-based microspheres [8]. Drug distribution in the sustained-release model is usually assumed uniform [9]; however, drug distribution profiles may vary among the microspheres actually. Understanding the drug distribution and

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pore structures throughout the microsphere precisely may provide significant insights into the structure building process and may facilitate the design of new improved sustained-release models. It can also contribute to the understanding with regard to the involvement of micro and nanostructures in drug release, at least in the early diffusion stage [10].

Various techniques have been used to characterize the pore structure of microspheres including theoretical approaches [11], scanning electron microscopy (SEM), mercury porosimetry, gas pycnometry, and gas adsorption. However, these techniques have certain limitations, and they are not readily applicable to porous microspheres. Electron and other microscopies are extensively used to characterize the microspheres; however, these techniques pose a challenge in quantifying the dimensions of structural features. Neither mercury porosimetry nor gas adsorption could be useful to characterize the closed pores. Gas adsorption methods can only be used to characterize the mesopores (2–50 nm). Using high pressure in the mercury porosimetry may crush the microspheres [12–14]. Although the drug distribution inside microspheres could be investigated using both confocal laser scanning microscopy (CLSM) and Fourier transform infrared spectroscopy, their resolutions could not fulfill the requirement of detailed drug distribution analysis [9]. Three-dimensional (3D) construction of overall drug distribution was successfully developed based on transmission electron microscopy combined with pixel number analysis. Yet, the sectioning and staining work was an onerous task [9,15].

Recently, the transmission hard X-ray microscopy (TXM) based on synchrotron radiation bridged the gap between the light and the electron microscopy [16–18]. Owing to the short wavelength and accurate X-ray optics, TXM may achieve a spatial resolution up to 26 nm now [19,20]. Due to hard X-ray's high penetration power and large depth of focus, transmission hard X-ray nano-computed-tomography (nano-CT) can provide 3D internal structure information of intact large samples with thickness up to 50 μm [21]. While under Zernike phase contrast mode, the high image contrast of nano-CT enables the visualization of 3D structures of low-absorbing samples such as biological tissues, organic materials, and soft materials [22–25].

However, to date nano-CT has not been fully exploited in the area of microspheres despite its potential for clarifying the relationships between delivery device formulation, structure, and release profile. In the present study, nano-CT was used to characterize the pore structure and drug distribution in PLGA microspheres encapsulating bovine serum albumin (BSA), fabricated by double-emulsion solvent evaporation method, with sodium chloride added into the internal aqueous phase as an osmotic agent [26]. The results of nano-CT were compared with the traditional methods including SEM and CLSM. Fundamental microstructural parameters such as size, shape, distribution of pores, and protein inside PLGA microspheres were obtained through 3D rendering of the PLGA microspheres. Surface pore interconnectivity and surface protein interconnectivity were calculated. Finally, the surface protein interconnectivity was correlated with the initial burst release among various tested formulations.

2. Materials and methods

Fluorescein isothiocyanate (FITC)-labeled BSA (FITC-BSA; Sigma Aldrich Co., St. Louis, MO, USA), PLGA (50:50, inherent viscosity 0.85 dL/g in chloroform; Birmingham Polymers Inc., Pelham, AL, USA), BSA (molecular weight 68 kDa; Roche Diagnostics, Indianapolis, USA), sodium alginate (Protanal LF 200 DL; FMC BioPolymer, Philadelphia, PA, USA), polyvinyl alcohol 124 (PVA-124, 98–99% hydrolyzed, Shanghai Zhanyun M&T Co. Ltd., Shanghai, China); Pluronic® F-68; BASF Corp., Ludwigshafen, Germany), and Pierce Micro Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) were purchased. All other reagents used were of analytical grade.

2.1. Preparation of PLGA microspheres

Microsphere-encapsulated BSA was prepared using modified double emulsion evaporation method ($W_1/O/W_2$). Briefly, 4 mL of 15% (w/v) PLGA in dichloromethane was emulsified using a high-speed homogenizer (IKA® ULTRA-TURRAX® T25 Digital Homogenizer; IKA-Werke GmbH & Co. KG, Staufen, Germany) with 0.3 mL of an internal aqueous phase containing 6 mg BSA, 3.6 mg sodium alginate, and 0.6 mg Pluronic F-68 (W_1). Different amounts of sodium chloride were added to the W_1 realizing theoretical sodium chloride:BSA mass ratios of 0.25:1, 0.5:1, 1:1, and 2:1 (B, C, D, and E groups, Table 1). The formulation without addition of sodium chloride was used as a control (A group, Table 1). The primary emulsion (W_1/O) was injected into an aqueous phase containing PVA-124 and calcium chloride, and then homogenized. The emulsion ($W_1/O/W_2$) was then transferred to a solvent extraction phase containing PVA-124 and calcium chloride, and the microspheres were hardened by stirring at 400 rpm for 3 h (IKA RW 16 Basic Overhead Stirrers; IKA-Werke GmbH & Co. KG). Microspheres were collected and washed three times with distilled water using centrifugation. Microspheres containing FITC-BSA were prepared using the same method described earlier, except that FITC-BSA was used instead of BSA.

2.2. Determination of BSA encapsulation efficiency

Accurately weighed dried microspheres (10 mg) were added into the centrifuge tubes containing 5.0 mL of 0.1 M sodium hydroxide/0.5% sodium dodecyl sulfate. The tubes were immersed in a shaker bath (Jiangsu Zhengji Instrument Co., Ltd., Jintan, China) maintained at 37.0 ± 0.5 °C and was shaken horizontally for 48 h. The amount of BSA in the supernatant was determined using Micro BCA Protein Assay Kit. The ratio of actual BSA content to theoretical BSA content was used to calculate the entrapment efficiency. All the tests were performed three times, and the results were reported as the means ± standard deviation (SD).

2.3. Electron microscopy access of particle morphology

After two cycles of sputter coating with gold, the surface and cross-sectional morphologies of PLGA microspheres were observed using an SEM (JSM-6330F; JEOL, Tokyo, Japan). After immobilizing the PLGA microspheres in an appropriate amount of aqueous solution containing 30% gelatin and 5% glycerin, cross-sectional samples were prepared using a freezing microtome (Leica CM1900 Cryostat; Leica Microsystems Nussloch GmbH, Nussloch, Germany) as described earlier [11]. The porosity and pore size of microspheres were determined using image analysis (Image-Pro Plus Software; Media Cybernetics, Bethesda, MD, USA). Porosity was defined as the fraction of the area of pore relative to the total area of cross-sections of microsphere. Six representative images were used for the calculation.

2.4. Characterization of drug distribution using CLSM

Drug distribution in PLGA microspheres (cross-sectional) was observed using CLSM (Laser Scanning Microscope 5100; Carl Zeiss, Oberkochen, Germany). The FITC was detected using an argon ion laser with an excitation wavelength of 488 nm and a 505–550 nm band-pass emission filter. All the images were obtained under the same resolution.