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Critical effect of freezing/freeze-drying on sustained release of FITC-dextran encapsulated within PLGA microspheres

Tae Hyoung Kim, Tae Gwan Park*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea

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Abstract

The cause of initial burst release of hydrophilic macromolecular drugs from biodegradable polymeric microspheres was identified. Poly(D,L-lactic-*co*-glycolic acid) microspheres encapsulating fluorescein isothiocyanate (FITC)-labled dextran was prepared by a double emulsion solvent evaporation method. The extent of initial burst release was examined by varying the formulation process conditions such as solvent evaporation, washing, freezing, and freeze-drying. Confocal microscopy was employed to analyze the underlying mechanism of burst release. The extent of burst release was gradually reduced after the repeated washing of embryonic microspheres before freeze-drying, indicating that FITC-dextran molecules entrapped within unhardened microspheres were slowly diffused out. However, freezing and subsequent drying processes of the embryonic microspheres resulted in much increased extent of burst release, suggesting that the initial burst release was primarily caused by the rapid diffusion of FITC-dextran through the microporous channels. Confocal microscopic analysis revealed that the freeze-drying process generated water-escaping micro-channels, through which the encapsulated molecules were presumably dumped out. Vacuum-drying was a good alternative choice in reducing the initial burst, compared to freeze-drying.

Keywords: Freezing/freeze-drying; Microspheres; Biodegradable polymer; Burst release

1. Introduction

Burst releases occurring in polymeric sustained delivery systems have been attributed to a variety of formulation factors involved in various steps of manufacturing processes, as well as to inherent physico-chemical properties of polymers and drugs (Langer, 1980; Peppas and Colombo, 1997). Primarily, surface adsorption of drug molecules on the matrix, uneven distribution of drug within the matrix, and porous matrix morphologies, are believed to be mainly responsible for the initial rapid release of drug upon incubation (Rafati et al., 1997; Takahata et al., 1998). The size and shape of the device also play an important role in determining the extent of burst release (Huang and Brazel, 2001). The burst release problem, often uncontrollable and unpredictable, is particularly acute for biodegradable small polymeric microspheres and nanoparticles encapsulating hydrophilic and macromolecular drugs such as peptides, proteins, and DNA (Johnson et al., 1996; Cohen et al., 1991). It has been understood that hydrophilic molecules encapsulated within the biodegradable polymeric microspheres tend to rapidly diffuse out upon incubation, when they are loosely encapsulated and/or surface adsorbed within or onto the microspheres that have

* Corresponding author. Tel.: +82-42-869-2621; fax: +82-42-869-2610.
E-mail address: tgpark@kaist.ac.kr (T.G. Park).

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a large surface area due to their small size. PLGA microspheres containing hydrophilic drugs have been popularly prepared by a double emulsion solvent evaporation method, which exhibits a wide array of microspheres with very different porous morphologies depending on the formulation parameters (Li et al., 2001; Kissel et al., 1996; Kim and Park, 1999). Therefore it has been difficult to pinpoint the cause of the burst release for different kinds of PLGA microspheres containing various hydrophilic drugs. In this study, it was hypothesized that a freezing and/or a freeze-drying process could be one of critical factors contributing to the initial burst release. When PLGA microspheres are prepared by the double emulsion solvent evaporation method, it is reasonable to postulate that the freeze-drying process inevitably generates water-escaping inter-connected microporous channels from the inner aqueous droplets to the outer surface. FITC-dextran, used as a model macromolecular drug, was encapsulated in PLGA microspheres to explore whether the freezing and/or freeze-drying process affect the initial burst. The extent of initial burst release from the microspheres was examined for the following various steps involved in the formulation processes: immediately after solvent evaporation, after washing, after freezing, and after freeze-drying. Confocal microscopy was employed to obtain the evidence of the micro-channel formation. In addition, an alternative drying process was proposed to minimize the burst release.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate labeled dextran (FITC-dextran, average MW 50,700) was obtained from Sigma Chemical Co. (St. Louis, MO). Poly(D,L-lactic-*co*-glycolic acid) having a 50/50 M composition of lactic/glycolic acid and MW 10,000 (RG502H) was purchased from Boehringer Ingelheim (Germany). Pluronic® L121 was the product of BASF (Parsippany, NJ). HPLC grade dichloromethane and methanol were obtained from Merck (Germany). Hydranal® composite 5, polyvinyl alcohol (PVA; 88% hydrolyzed, MW 25,000) were the product of Aldrich (St. Louis, MO). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of microspheres

PLGA microspheres containing FITC-dextran were fabricated using a water-in-oil-in-water (w/o/w) double-emulsion solvent evaporation technique (Kim and Park, 1999; Crotts et al., 1997). Briefly, 300 μl of aqueous FITC-dextran solution (100 mg/ml) was emulsified in 5 ml of dichloromethane containing PLGA (20%, w/v) and Pluronic® L121 (2%, v/v) as a surfactant by homogenization (Powergen 700, Fischer Scientific, Germany) for 60 s. The primary emulsion was added to 150 ml of 1% polyvinylalcohol solution saturated by dichloromethane, and the solution was homogenized for 90 s to get a secondary emulsion. The secondary emulsion was rapidly stirred and the solvent was allowed to evaporate at room temperature for 3 h. FITC-dextran loaded microspheres were collected by filtration and washed with deionized water. The microspheres were then freeze-dried under vacuum (−49 °C and <1 μmHg) using a standard freeze-drier (Ilshin, Korea) after freezing in liquid nitrogen or dried in a vacuum oven (Precision Scientific, Chicago, IL) at room temperature. Blank microspheres (FITC-dextran free) were also prepared by the solvent evaporation method described above.

2.2.2. In vitro release studies

After the solvent evaporation, unhardened microspheres were first washed with 150 ml of deionized water and collected by filtration. Thirty milligrams of microspheres in wet weight was used to test the effect of the number of washing on the burst release. Each washing time was 30 min and the volume of deionized water for washing was 150 ml. For sustained release experiments, 13 mg of FITC-dextran loaded microspheres was suspended in a 15 ml centrifuge tube containing 5 ml PBS (pH 7.4) which also contained 0.02% (w/v) sodium azide and 0.02% (w/v) Tween 20. The suspension was incubated at 37 °C under continuous agitation. At predetermined time intervals, microspheres and supernatant were separated by centrifugation at 5000 rpm for 10 min. The clear supernatant of the release medium (4 ml) was withdrawn and the same volume of fresh buffer solution was replenished. The amount of FITC-dextran released was measured using a fluorescence spectrometer (RF-5301PC, Shimadzu, Japan) at an

excitation and an emission wavelength of 493 and 515 nm, respectively.

2.2.3. Confocal laser scanning microscopy

Confocal laser scanning microscopy was used to investigate the distribution of FITC-dextran within the microspheres after the burst release. The FITC-dextran loaded microspheres, after incubating in the release medium for 1 day, were placed onto a glass slide, and the fluorescent image was taken. In order to observe the diffusion-in process of FITC-dextran into the blank microspheres, 15 mg of freezed, vacuum-dried, or freeze-dried blank micropsheres were hydrated with 1 ml of PBS, and then incubated with 1 ml of FITC-dextran solution (2 mg/ml). After incubating for 1 day at 37 °C under continuous agitation, the microspheres were separated by centrifugation at 5000 rpm for 10 min. After washing with 5 ml of PBS, the distribution of diffused FITC-dextran within the microspheres was observed by Carl Zeiss LSM 5100 confocal laser scanning microscopy (Germany).

3. Results and discussion

3.1. The burst release fraction as a function of number of washing

The number of washing after the solvent evaporation was examined to see how it affected the initial burst release. In many previous studies, it was assumed that the initial burst release was primarily caused by the presence of encapsulated drug molecules in the vicinity of surface region of the hydrated microspheres (Calis et al., 1995). To test this speculation, hydrated microspheres, just retrieved from the solvent evaporation process, were washed as a function of the number of washing time. These wet microspheres without further freeze-drying were directly subjected to the release experiment. The loading amount of FITC-dextran within PLGA microspheres was $0.24 \pm 0.02\%$ (w/w) before washing and $0.18 \pm 0.04\%$ (w/w) after seven cycles of washing. Fig. 1 shows the fractional extent of burst release for the microspheres after 1 day incubation with increasing number of washing. The initial release fraction at each washing cycle gradually decreases from $33.0 \pm 8.4\%$ to $11.6 \pm 0.3\%$, but it tends to stabilize

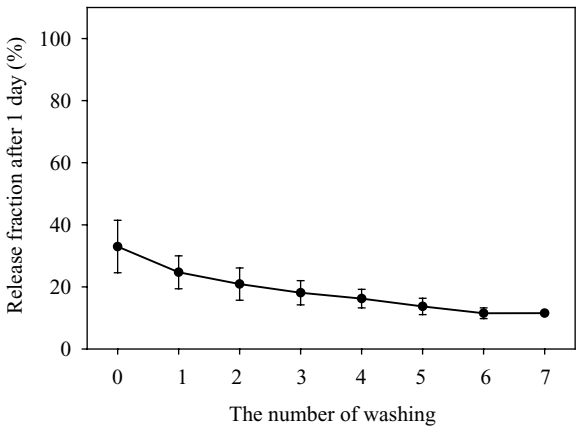

Fig. 1. The burst release fraction of FITC-dextran loaded microspheres as a function of number of washing ($n = 3$). The burst release amount was determined after 1 day incubation.

with increasing number of washing, revealing that the extensive washing process certainly reduced the degree of burst release to some extent. This suggests that the initial burst release of FITC-dextran from unfreeze-dried, hydrated, and insufficiently washed microspheres was mainly caused by rapid diffusion of loosely entrapped FITC-dextran preferentially located in the surface region. After repeated washing, it was likely that FITC-dextran entrapped within the inner region continuously leached out through the preformed pores in the release medium.

3.2. Water content as a function of freeze-drying time

The hydrated microspheres after washing seven times were separated by a 0.45 μm membrane filter and freeze-dried. Their water contents were determined as a function of freeze-drying time. The water content of hydrated microspheres was measured in triplicate by a Karl–Fischer titration method. The relationship between the water content and the freeze-drying time is shown in Fig. 2. It can be seen that the initial water content of $24.7 \pm 0.4\%$ before freeze-drying greatly decreases within the first 2 h period and then gradually decreases to a plateau value, $2.3 \pm 1.0\%$ at 24 h. During the initial 10 min period, the initial water content value was sharply reduced. As the freeze-drying process continued, the water content of the microspheres was further reduced to

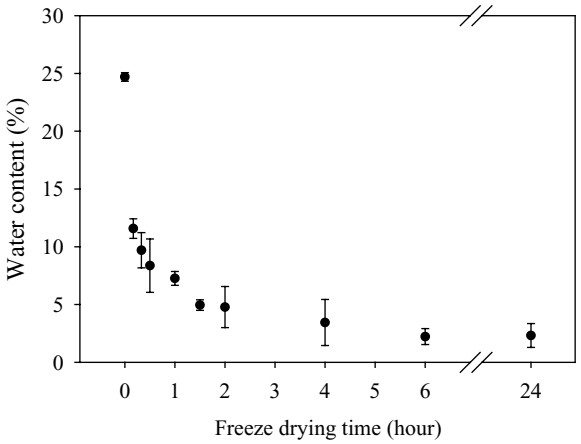

Fig. 2. Water content of FITC-dextran loaded microspheres as a function of freeze-drying time ($n = 3$).

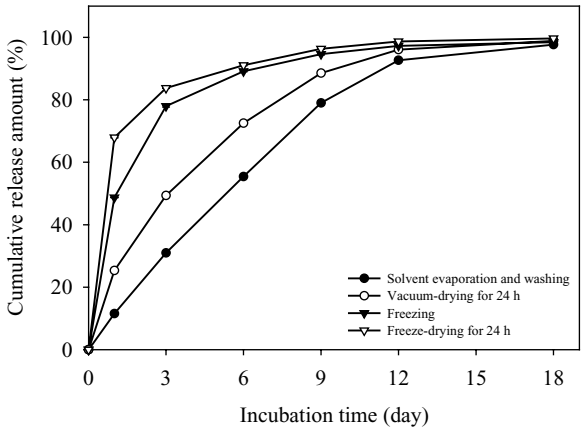

Fig. 3. Release profiles of FITC-dextran loaded microspheres treated with different processes ($n = 3$).

$2.2 \pm 0.7\%$ at 6 h and then stabilized thereafter. This suggests that the fraction of freezable (free) water within the microspheres could be easily removed, but the fraction of bound water was more slowly sublimed and still remained after freeze-drying for 24 h. Presumably, pores and their interconnected channels within the frozen microspheres were likely to be filled with freezable free water molecules, whereas their walls and entrapped FITC-dextran molecules were tightly interacted with unremovable bound water molecules. The water content of 24.7% for the wet microspheres implies that the fractional volume of water-filled pores and their interconnections relative to the overall volume of the wet microspheres was approximately about 24.7%.

A freezing step is a prerequisite for the following freeze-drying process. This freezing process was likely to induce the formation of ice-crystals of free water molecules present within the pores and their interconnected channels. The ice-crystals were then sublimed under reduced pressure to obtain dried microspheres. Thus it is highly probable that the porous morphology of the resultant microspheres was dependent on the imprinted dimension and shape of ice-crystals developed during the freezing process. The increased volume fraction of ice upon freezing was likely to induce the enlargement of liquid water-filled pore dimensions with a concomitant cracking of PLGA polymer walls surrounding the pores (Passerini and Craig, 2001). Because PLGA polymer was in a glassy state under the liquid nitrogen temperature, the PLGA polymer walls surrounding the water-filled pores could develop small cracks, producing more micro-channels upon freezing.

After the solvent evaporation and washing process, the wet microspheres showed $11.6 \pm 0.3\%$ of initial release as shown in Fig. 1, but after one cycle of freezing-thawing, they demonstrated $48.7 \pm 0.0\%$ as shown in Fig. 3. This suggests that enlarged pores with the development of inter-connected channels might be responsible for the increased extent of initial release. Freeze-drying is a unit operation process to remove water molecules from an ice state to a vapor state under reduced pressure (Overcashier et al., 1999). This process was also likely to induce the additional change in pore morphology of the microspheres. Water vapor molecules from ice crystal particles, embedded throughout the polymer matrix, should escape to the outer surface of the microspheres. This water removal process might generate further micro-channel pores from the loci of ice crystals to the surface. In particular, if ice crystal particles were developed within isolated and closed pore domains, the freeze-drying step would create the micro-channels around them.

3.3. Release profiles of FITC-dextran loaded microspheres treated with different processes

Fig. 3 shows the release profiles of FITC-dextran from the different microspheres that were incubated in the medium after undergoing various treatment

processes: solvent evaporation and washing, freezing, freeze-drying, and vacuum-drying. Three samples were used to determine the release fraction for each process. The overall release profiles as well as the extent of burst release were significantly affected depending on how the microspheres were treated. As expected, the microspheres undergoing the freezing or the freeze-drying process show very high initial burst release fractions with rapid release kinetic profiles, while those experiencing only the solvent evaporation and washing process without freezing or freeze-drying demonstrate a burst free sustained release profile over a period of 12 days. The solvent evaporated and washed microspheres in a hydrated state exhibit a near zero-order release kinetic profile that has been very difficult to achieve for hydrophilic macromolecular drugs. Hence it is reasonable to say that the wet microspheres retrieved prior to freezing and freeze-drying processes had a diffusion-controlled FITC-dextran release that occurred through the pre-formed pores and their connections. Encapsulated FITC-dextran molecules located within the inter-connected pores and channels would preferentially diffuse out without initial burst release, whereas those within rather isolated pores would diffuse out more slowly. This mechanism took place from the very early stage of incubation. It should be noted, however, that the desirable linear release profile achieved from the hydrated microspheres had very limited practical applications, because PLGA microspheres must be dried as a dosage form. In contrast, upon freezing and freeze-drying, the same microspheres, but in a dry state, demonstrate significantly increased extents of burst release, supporting that both freezing and freeze-drying processes are the main cause for the observed burst releases. As mentioned earlier, the changes in pore size, geometry, and pore interconnection possibly occurred during the freezing and subsequent drying steps could be attributed to the cause of the burst release. The local presence of FITC-dextran near the surface of microspheres with a concomitant desorption mechanism appear to play a minor role in causing the burst release. To minimize the extent of burst release, the solvent evaporated and washed microspheres were directly dried under vacuum at room as an alternative way to avoid the detrimental effect of freezing and freeze-drying on the burst release as well as on the overall release profile. A much better release profile of FITC-dextran with significantly reduced extent of burst release can be seen from vacuum-dried microspheres than from frozen and freezed dried microspheres. This result emphasizes the importance of drying methods for achieving desirable sustained release profiles of many hydrophilic drugs. The vacuum-drying method was initially thought to produce aggregated microspheres in comparison to the freeze-drying method, because the vacuum-drying temperature (room temperature) was in the range of the glass transition temperature (T_g) of the wet microspheres. The resultant microspheres, however, were not aggregated and they were free flowing. Scanning electron microscopy revealed that average sizes of freeze-dried and vacuum-dried microspheres were $36.3 \pm 16.2\,\mu m$ and $37.0 \pm 13.2\,\mu m$ in diameter, respectively. No significant differences in size, shape, and surface smoothness could be seen between the two microspheres, proving that the vacuum-drying is a good alternative way for drying of the PLGA microspheres containing FITC-dextran.

3.4. Confocal fluorescence images with different processes FITC-dextran loaded microspheres

Confocal microscopic images of the microspheres were taken to directly visualize the distribution of FITC-dextran throughout the matrix. Fig. 4 shows the fluorescent images of freeze-thawed, freeze-dried, vacuum-dried microspheres encapsulating FITC-dextran after incubating for 1 day in the release medium. It also shows the flurorescent images of freeze-thawed, freeze-dried, and vacuum-dried blank microspheres after incubating in the solution containing FITC-dextran for 1 day. In Fig. 4, the left panel shows the images for the three different microspheres after the release experiment and the right panel shows the images for the blank microspheres after the uptake experiment. The wet microspheres, just retrieved after the solvent evaporation and washing, had relatively homogeneous distribution of FITC-dextran throughout the matrix without showing any localized distribution near the surface. In the release experiment, it can be seen that vacuum-dried microspheres still maintain the uniform distribution of FITC-dextran, whereas freeze-thawed and freeze-dried microspheres have isolated reservoir droplets of FITC-dextran. The scattered fluorescent droplets are more clearly

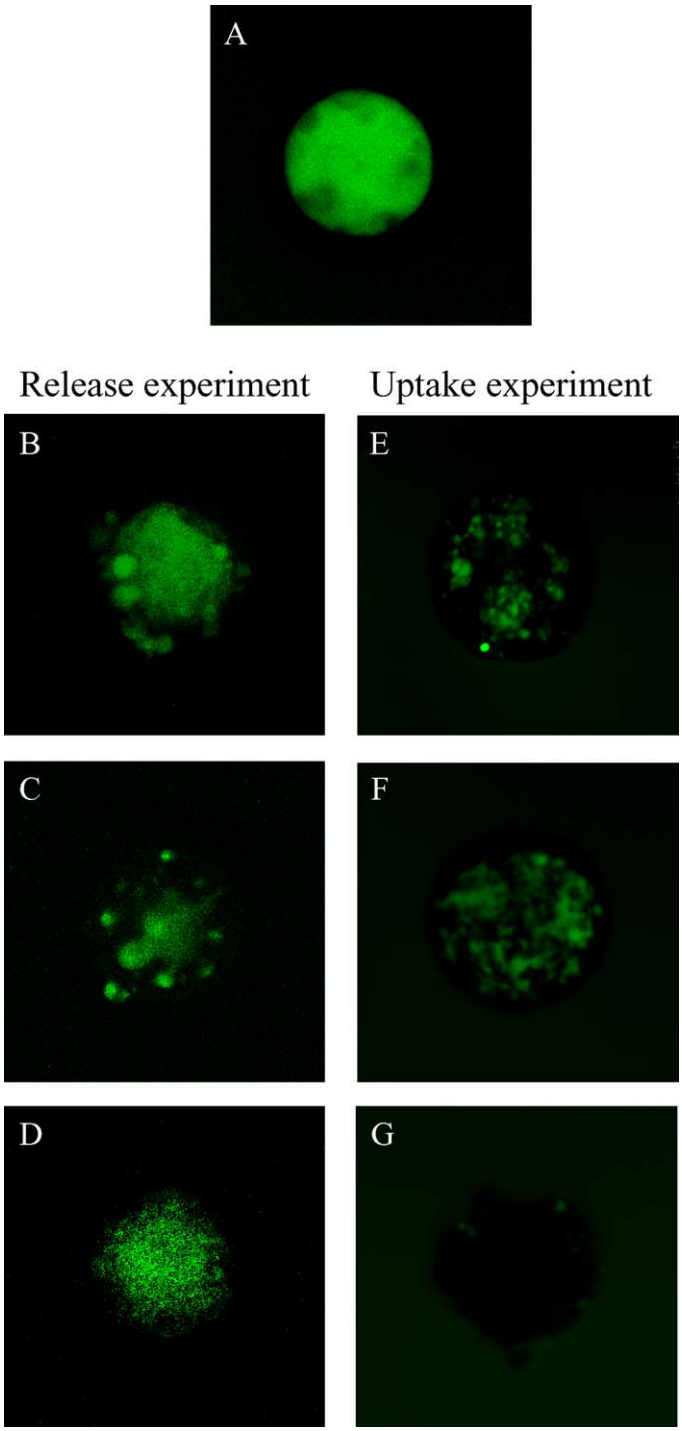

Fig. 4. Confocal fluorescence images of (A) solvent evaporation and washed, (B) freeze-thawed, (C) freeze-dried, and (D) vacuum-dried FITC-dextran loaded microspheres after 1 day incubation (the left panel). The right panel is those of (E) freeze-thawed, (F) freeze-dried, and (G) vacuum-dried blank microspheres incubated in FITC-dextran solution for 1 day.

visualized for the freeze-dried microspheres. In the uptake experiment, FITC-dextran could diffuse more readily into the freeze-dried microspheres than the vacuum-dried microspheres. The vacuum-dried microspheres demonstrate negligible fluorescent staining inside of the matrix. From the confocal microscopic images of FITC-dextran distribution within the microspheres, it is evident that freezing and freeze-drying processes generated micropores and their interconnected channels throughout the polymer matrix. These porous channels existing within freeze-thawed and freeze-dried microspheres were large enough for FITC-dextran to freely diffuse-in and diffuse-out the microspheres. On the other hand, the vacuum-dried microspheres had a limited diffusion of FITC-dextran in the release and uptake experiments, supporting that the microporous channels were not readily formed during the vacuum-drying process. These confocal microscopic results suggest that the initial burst release of FITC-dextran from the microspheres was mainly caused by the formation of microporous and interconnected channels during freezing and freeze-drying processes. However, scanning electron microscopy observation did not provide clear evidence for the development of surface micropores before and after freezing and freeze-drying, possibly due to their nano-scale dimension. To further validate the formation of the microporous channels, more rigorous structural characterization using atomic force microscopy would be necessary. Certainly, there would be other important formulation factors in affecting the burst release in vastly different ways; for example, water/methylene chloride ratio, polymer concentration, drug loading amount, and particle size (Yang et al., 2001). The present study aimed to elucidate the mechanism of burst release only for the FITC-dextran encapsulated PLGA microspheres prepared by the double emulsion solvent evaporation method under fixed formulation conditions. There have been few studies emphasizing the importance of freezing/freeze-drying on the burst release of hydrophilic drugs.

4. Conclusion

We have shown that the development of micropores and their interconnections as a result of ice crystallization and sublimation during freezing and drying process was one of critical factors in determining the extent of burst release often observed for biodegradable microspheres. Vacuum-drying could be an alternative method for achieving a sustained release profile with minimizing the extent of burst release.

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