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Particle Size and Temperature Effect on the Physical Stability of PLGA Nanospheres and Microspheres Containing Bodipy

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ABSTRACT

The purpose of this study was to investigate the effect of particle size, storage temperature, and duration of storage on the physical stability and morphology of polylactic-co-glycolic acid (PLGA) nanospheres and microspheres. PLGA nanospheres and microspheres containing the fluorescent dye, Bodipy, were prepared in varying sizes by controlling the method and degree of agitation during the emulsification phase of preparation. Mean diameters of the particles were measured by dynamic light scattering. To evaluate the effect of storage temperature and duration of storage on the extent of aggregation, nanospheres and microspheres were stored at 4°C, 25°C, 37°C, and 50°C for 6 days and then monitored using both confocal and scanning electron microscopy. The mean ± SD diameters of PLGA particles containing Bodipy were: 266.9 ± 2.8, 351.6 ± 1.8, 988.8 ± 14.1, and 1865.9 ± 67.0 nm. The extent of aggregation of the particulate delivery system decreased as the mean diameter increased, and increased as the storage temperature increased. The maximum extent of aggregation was observed with the smallest (266 nm) nanospheres. Microspheres did not aggregate. The aggregation of nanospheres was significantly reduced by introducing an additional evaporation step during preparation, suggesting that migration of residual dichloromethane from within the nanospheres may have dissolved the PLGA on the surface. The extent of aggregation of nanospheres increased as the temperature was increased from 4°C to 50°C, and decreased as particle size increased. To avoid aggregation, PLGA nanospheres should be stored at 4°C.

KEYWORDS: nanospheres, microspheres, Bodipy, temperature, aggregation.

INTRODUCTION

Nanospheres and microspheres have been extensively used to deliver a wide range of drugs as they can protect the drug from metabolizing enzymes, sustain the release, be administered orally or injected locally, and target specific tissues by incorporating surface ligand moieties.[1-8] A common polymer used in the formulation of nanospheres and microspheres is the biodegradable, biocompatible polymer, polylactic-co-glycolic acid (PLGA).[5-9] This research is a part of the overall study of the effect of the mean diameter of the particulate delivery systems on the cellular accumulation, cytotoxicity, and efficacy of paclitaxel. During these studies, there was evidence that the temperature of storage is important in maintaining the physical integrity of particulate delivery systems.

There have been numerous publications on the rate and extent of chemical degradation of PLGA used in various drug delivery systems, including the factors that influence the kinetics of this reaction.[9,10] The factors that influence the chemical degradation of PLGA are well known and include polymer molecular weight, ratio of lactic to glycolic acid in the co-polymers, polymer-drug ratio, environmental temperature, pH, and geometry of the delivery system.[11] PLGA undergoes random hydrolysis to oligomers, then ultimately to the nontoxic, monomeric units, lactic and glycolic acids. Oligomers hydrolyze more rapidly than the higher molecular weight parent polymer due to the presence of more terminal carboxylic acid groups per unit molecular mass.[9,12-19] Lactic and glycolic acids occur naturally in the human body and are easily eliminated through the glycolytic pathway as carbon dioxide and water. It has been shown that PLGA nanospheres and microspheres have a shelf-life of more than 3 months (PLGA 50:50, 0.63 dL/g).[5] However, to our knowledge, the influence of storage temperature, duration of storage, and particle size on the physical stability and morphology of nanospheres and microspheres has not been reported.

During preparation, PLGA particulate delivery systems are lyophilized and, although compactly arranged, can be readily resuspended in aqueous media after being stored at 4°C. However, we observed that particle size and storage temperature are important in maintaining the integrity of these delivery systems. Therefore, the purpose of this research was to study the effect of storage temperature, storage duration, and mean diameter of particles on the physical stability and morphology of PLGA particles, and on their potential to be redispersed after storage. To observe potential changes in the extent of aggregation using confocal microscopy, PLGA nanospheres and microspheres were prepared containing a lipophilic, green fluorescent dye, Bodipy Fl C5 (λ_{ex}:495 nm; λ_{em}:512 nm). In

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addition, scanning electron microscopy (SEM) was used to study changes in aggregation, coalescence, and solid-state surface morphology of the PLGA particles.

Materials and Methods

Materials

PLGA (50:50) with inherent viscosity of 0.69 dL/g (Lot No. 112-66-1) was obtained from Birmingham Polymers Inc (Birmingham, AL). Polyvinylalcohol (PVA), sodium chloride, sodium dihydrogen phosphate, and disodium hydrogen phosphate were obtained from Sigma Chemicals (St Louis, MO). BODIPY Fl C_5 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid, D-3834) was purchased from Molecular Probes (Eugene, OR).

Methods

Preparation of Nanospheres and Microspheres Containing Bodipy

Nanospheres containing Bodipy were prepared using the conventional emulsion-evaporation technique.[20,21] Initially a 500-µg/mL solution of Bodipy was prepared in methanol. PLGA (90 mg) was dissolved in 3 mL of dichloromethane and 0.1 mL of Bodipy (50 µg) in methanol was added. This solution was then emulsified for 60 seconds with 25 mL of 1.5% wt/vol PVA solution using a microtip probe sonicator at 36 W (Misonix Sonicator 3000 with microtip probe, Misonix Inc, Farmingdale, NY). Two sizes of nanospheres were prepared by emulsifying the 2 phases for 60 seconds at 36 or 10 W using a Misonix sonicator, while emulsification for 60 seconds using a Sorvall Omni-Mixer (Sorvall Instruments, Norwalk, CT) at 3 and 1 W formed larger microspheres. The dichloromethane was evaporated by stirring overnight, and the suspension ultracentrifuged at 140 000*g* for 25 minutes at 4°C (34 500 rpm using 50.2 Ti rotor). The remaining pellet was resuspended in water by sonicating for 30 seconds and washed twice with water. The washings were centrifuged at 130 000*g* (33 000 rpm) for 20 minutes at 4°C, and the pellet was resuspended by sonication and maintained at -80°C for 2 hours before being lyophilized for 36 hours. The resulting nanospheres were then stored in a dessicator at 4°C until used.

Characterization of Nanospheres and Microspheres

Determination of Particle Size and Size Distribution Using Dynamic Light Scattering

Nanospheres or microspheres (0.5 mg) were dispersed in 3 mL of water by sonication and added then to a 3-mL cuvette. The mean particle size ± SD, and zeta potential of each batch of particles were determined using the Zeta Plus dynamic light scattering particle size analyzer (Brookhaven Instrument Corp, Holtsville, NY).

Effect of Storage Temperature on the Aggregation of PLGA Particles of Varying Size

The Effect of Particle Size on the Changes in Morphology of PLGA Nanospheres and Microspheres Stored at 37°C

To establish if there was an effect of particle size on the morphology of PLGA particles containing Bodipy when stored at 37°C, nanospheres and microspheres were prepared with the following approximate diameters: 250 nm, 350 nm, 1 µm, and 2 µm. Samples of the PLGA-Bodipy particles were incubated at 37°C in 20-mL glass scintillation vials. After 6 days, 0.5 mg of these particles was suspended in 3 mL of water and visualized using a Zeiss confocal microscope (LSM 410 Confocal Laser Scanning Microscope [Goettinger, Germany]) with 1 filter at 488 nm and another cut-off filter at 515 nm. The duration of storage, 6 days, was selected based on previous time-dependent aggregation studies on particles in the same size range.

Effect of Storage Temperature on Nanosphere Aggregation

To study the effect of temperature on the extent of aggregation during storage, the smallest Bodipy-PLGA nanospheres (266 nm, 5 mg) contained in scintillation vials were incubated at 4°C, 25°C, 37°C, and 50°C for 6 days. The extent of aggregation of these nanospheres was monitored after 6 days using both confocal microscopy and SEM. In addition, differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) thermograms of the Bodipy-PLGA particles at 0 and after 6 days were obtained using a Shimadzu DSC-50 and TGA-50 (Shimadzu Corporation, Kyoto, Japan) and compared with control pure PLGA polymer. The DSC and TGA thermograms were obtained after sealing 1 mg of particles, which had been stored at each temperature, in aluminum pans and heating at a rate of 10°C/min to 350°C in an atmosphere of nitrogen (20 mL/min).

Effect of PVA Concentration and Residual Dichloromethane on the Extent of Aggregation

To investigate if PVA influenced the extent of aggregation, Bodipy-PLGA nanospheres (300 nm) were prepared using the previously described protocol, except that the organic phase was emulsified with aqueous PVA solutions of 3 concentrations (0.75%, 1.5%, and 2.5% wt/vol). Samples (5 mg) from these 3 batches of nanospheres were stored in scintillation vials at 4°C, 25°C, 37°C, and 50°C for 6 days. After 6 days, the extent of aggregation and morphology of the nanospheres was observed using confocal microscopy and SEM, and their DSC and TGA thermograms were recorded.

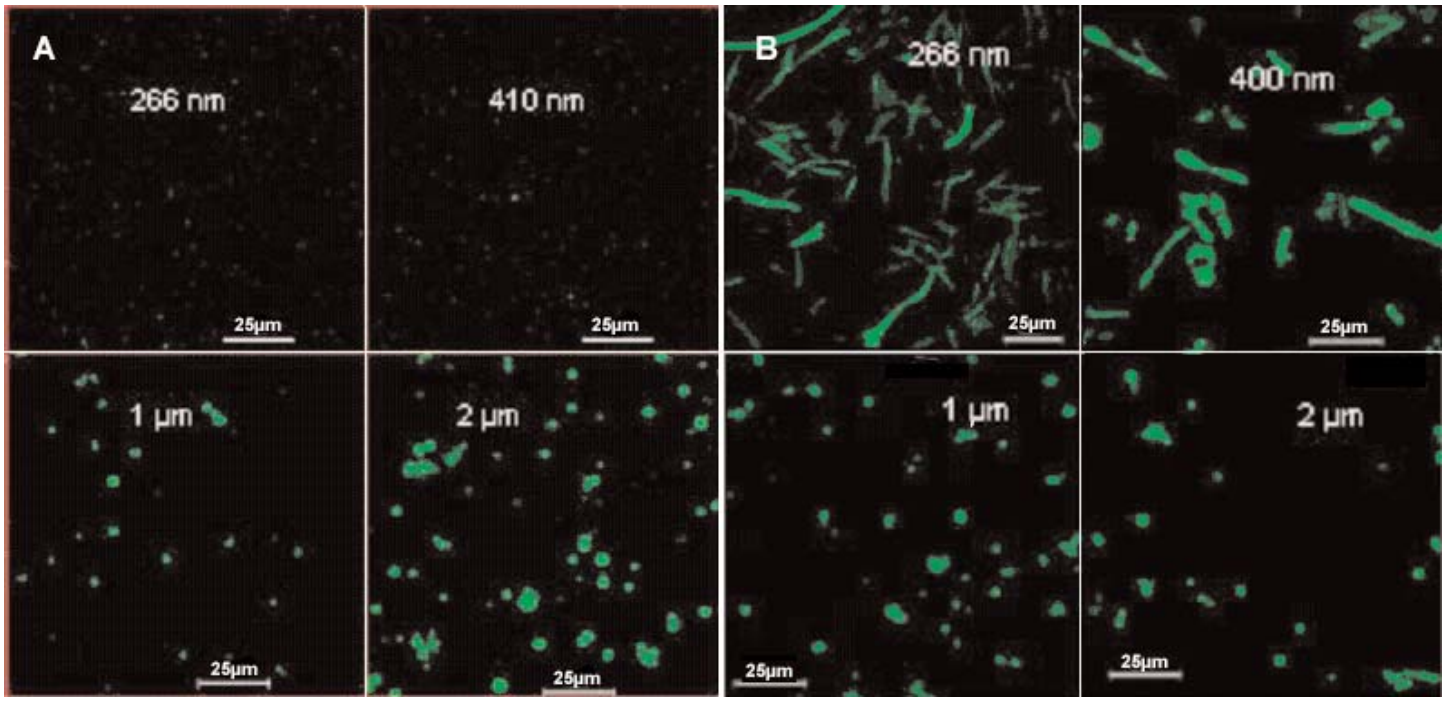

Figure 1. Effect of diameter of Bodipy-PLGA particles on the degree and extent of aggregation (A) before and (B) after storage at 37°C for 6 days. Particle sizes are stated in each figure.

To test if the aggregation of nanospheres may have been due to the residual dichloromethane within the particles, the particles were prepared according to the above protocol, except the suspension of nanospheres was placed in a rotary evaporator for 2 hours at 27°C after stirring overnight. The nanospheres were then washed to remove PVA and lyophilized as described previously. After lyophilization, samples (5 mg) of these nanospheres (266 nm), prepared using an additional rotavap step, were stored in scintillation vials at 37°C for 0, 1, and 2 days. To evaluate whether changes had occurred, nanospheres were then resuspended and observed using confocal microscopy, and the DSC and TGA thermograms were obtained. Nanospheres that had not been subjected to the additional evaporation step were used as control.

Results and Discussion

Preparation and Characterization of Various-Sized BODIPY-PLGA Particles and the Effect of Storing the Particles at 37°C on Aggregation

The mean diameter and SD of the batches of lyophilized Bodipy-PLGA particles (Figure 1A) were 266.9 ± 2.8, 351.6 ± 1.8, 988.8 ± 14.1, and 1865.9 ± 67.0 nm with corresponding Bodipy content of 0.026%, 0.027%, 0.028%, and 0.030% wt/wt, respectively. The particles were formulated using the standard emulsion-evaporation method and then lyophilized. After lyophilization, all particles were spherical with smooth surfaces. SEM photomicrographs of Bodipy-PLGA control (prior to storage) particles, independent of size, illustrated that all nanospheres and microspheres were discrete entities and compactly arranged after lyophilization

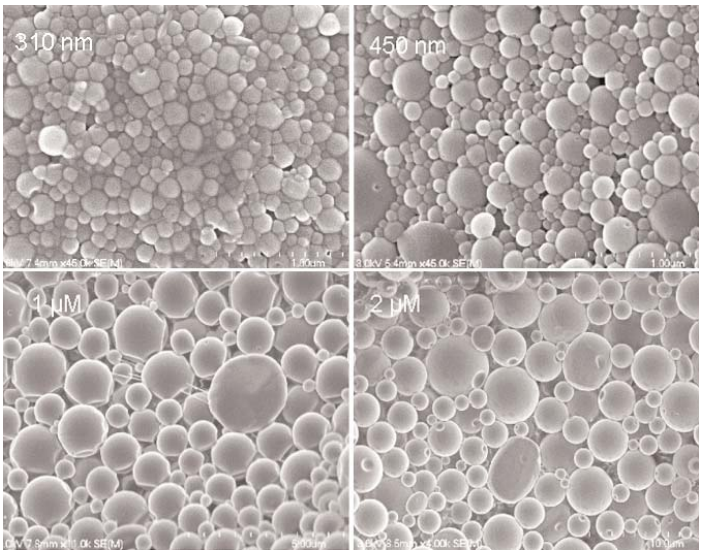

Figure 2. SEM photomicrographs of discrete, yet compactly arranged, different sized, lyophilized PLGA nanospheres and microspheres before storage at elevated temperatures.

(Figure 2). Of importance, additional SEM studies indicated that lyophilization caused the PLGA particles to align in intimate contact as fibers (Figure 3). This finding indicates that lyophilization did not cause aggregation of only the nanospheres. In addition, the alignment of fibers may facilitate the subsequent aggregation of particles when stored at elevated temperatures (Figure 1B). Confocal microscopy revealed that, after storage at 37°C for 6 days, significant aggregation of nanospheres had occurred. Of importance, microspheres did not aggregate when stored under these conditions (Figure 1B). More specifically, the extent of aggregation of PLGA particles stored at 37°C decreased as the particle size of the

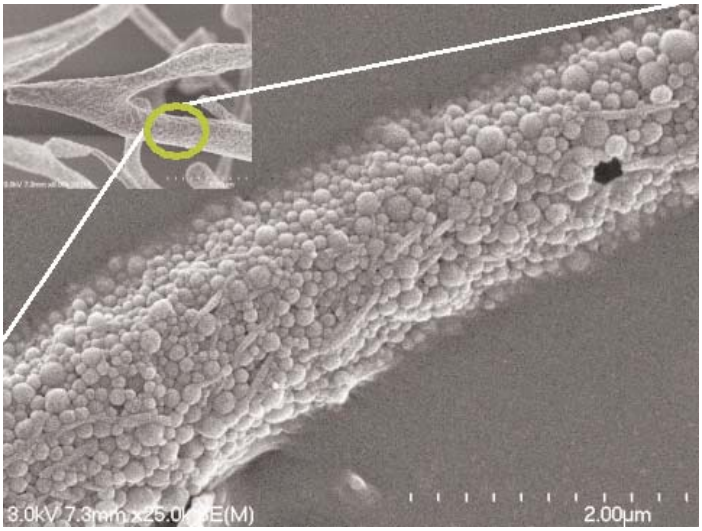

Figure 3. SEM photomicrographs of fibrous alignment of PLGA nanospheres, after lyophilization.

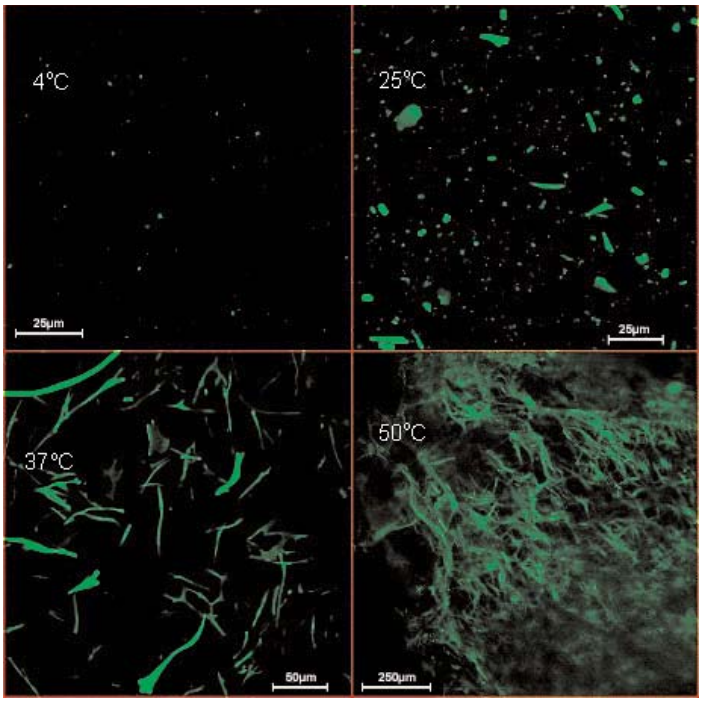

Figure 4. Effect of temperature of storage on the extent of aggregation of 266-nm PLGA 50:50 (IV: 0.69 dL/g) nanospheres containing Bodipy, after storing at 4°C, 25°C, 37°C, and 50°C for 6 days.

delivery system increased (Figure 1B). Subsequent experiments at 37°C demonstrated that aggregation of the smallest nanospheres (266 nm) began at day 1, and they were completely coalesced by day 2 (Figures 4 and 5).

Effect of Storage Temperature on the Extent of Aggregation of BODIPY-PLGA Nanospheres

As aggregation was most pronounced with the smallest Bodipy-PLGA nanospheres (266 nm) stored at 37°C, samples of these particles were used to subsequently study the effect of storage temperature (4°C, 25°C, 37°C, and 50°C) on their physical stability over a period of 6 days (Figure 3).

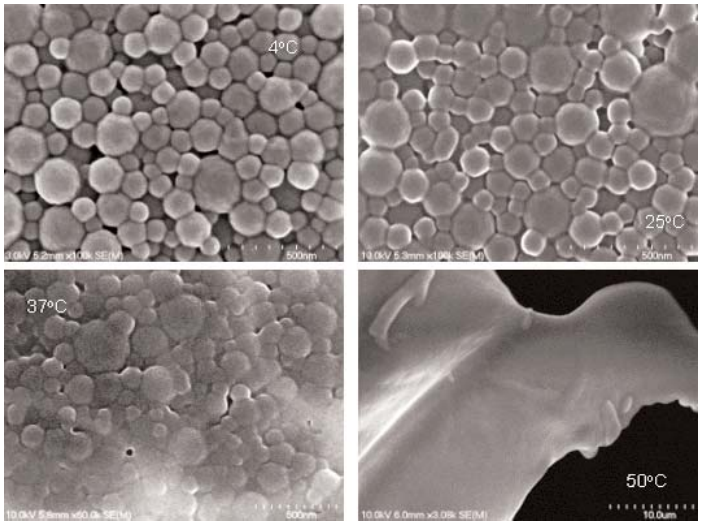

Figure 5. SEM photomicrographs illustrating the effect of temperature of storage on the extent of aggregation of 266-nm Bodipy-PLGA nanospheres after storing at 4°C, 25°C, 37°C, and 50°C for 6 days.

When examined using confocal microscopy, no aggregation of the nanospheres was observed after being stored at 4°C for 6 days. Some aggregation of particles was observed after 6 days when stored at 25°C. As the storage temperature was increased to 37°C, nanospheres had aggregated into larger (~50-µm) needles that could not be redispersed even after extensive sonication. After storage for 6 days at 50°C, the nanospheres coalesced into a solid, elastic mass. These observations were confirmed using SEM (Figure 4). Figure 3 also demonstrates that, after lyophilization, the nanospheres arrange to form a compact, fibrous network. However, these nanospheres are readily redispersible when stored correctly. Again, DSC and TGA thermograms of particles containing Bodipy did not indicate changes in either chemical stability of the polymer or thermal properties such as glass transition or melting temperature, indicating the absence of chemical degradation or polymer-dye interaction (Figure 5). Figure 6 illustrates that the DSC thermograms of Bodipy-PLGA particles after storage at 4°C, 25°C, 37°C, and 50°C for 6 days that PLGA was not significantly affected by the temperature. The glass transition temperature (Tg) remained relatively constant (onset 37.98°C; endset 45.32°C, and glass transition: -0.691 mW) after storage at each temperature. Charring of the sample and decrease in mass that occurred between 250°C and 320°C indicated chemical decomposition of the polymer occurred in all samples above 300°C. The thermogravimetric analysis did not show any evidence of entrapped water. The main difference is the prolonged endothermic phase, from heating the samples above 200°C for samples stored at 4°C. This observation was less pronounced for samples stored at 25°C and 37°C and absent in the samples stored at 50°C (Figure 6). We conclude this observation may be due to the evaporation of entrapped dichloromethane (becquerel [bp] 39.7°C) in samples stored at lower tempera-

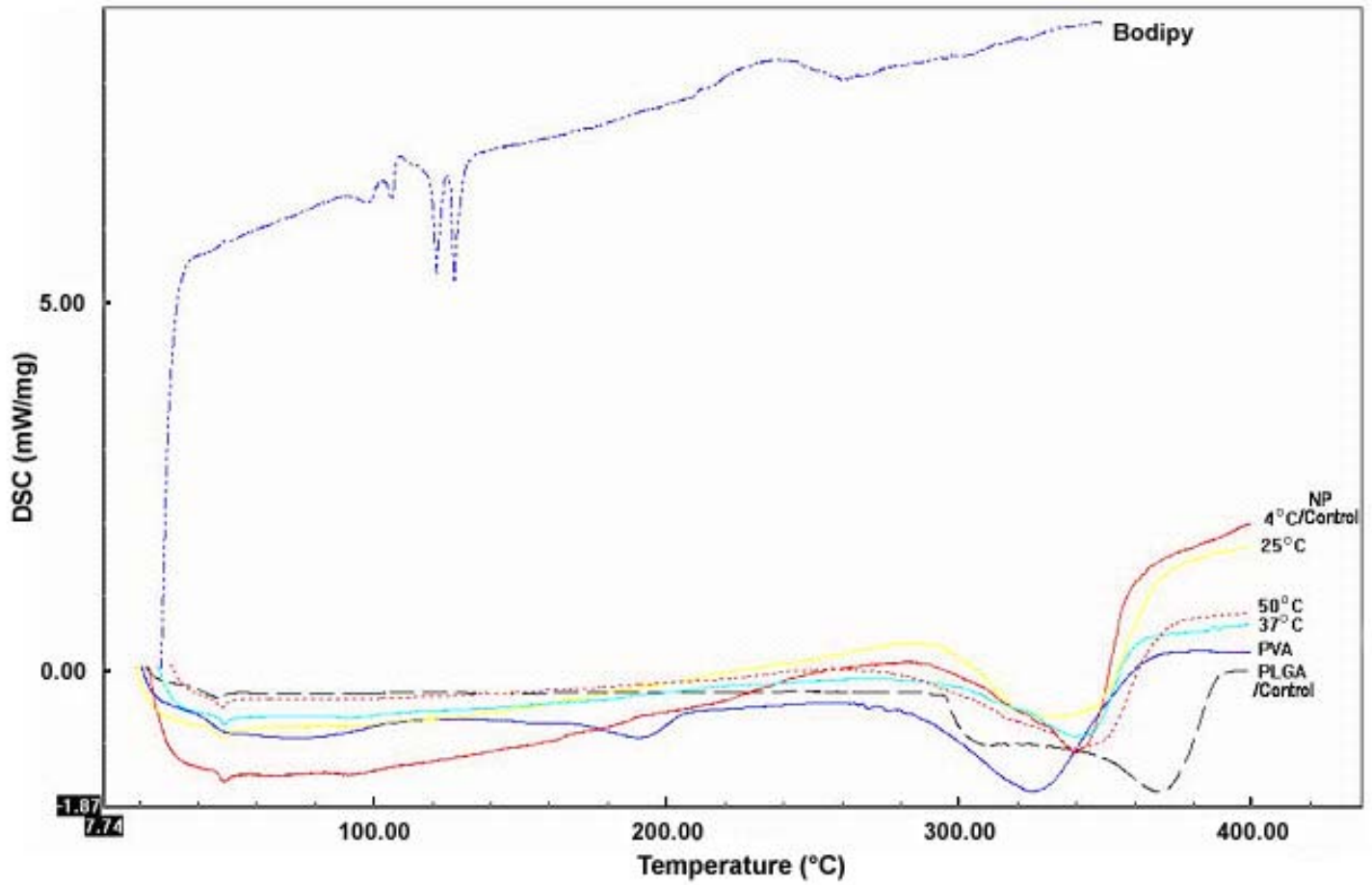

Figure 6. DSC thermograms of 266-nm PLGA nanospheres containing Bodipy after storing for 6 days at 4°C (nanosphere control), 25°C, 37°C, and 50°C. DSC thermograms of pure PLGA, PVA, and Bodipy are used as controls.

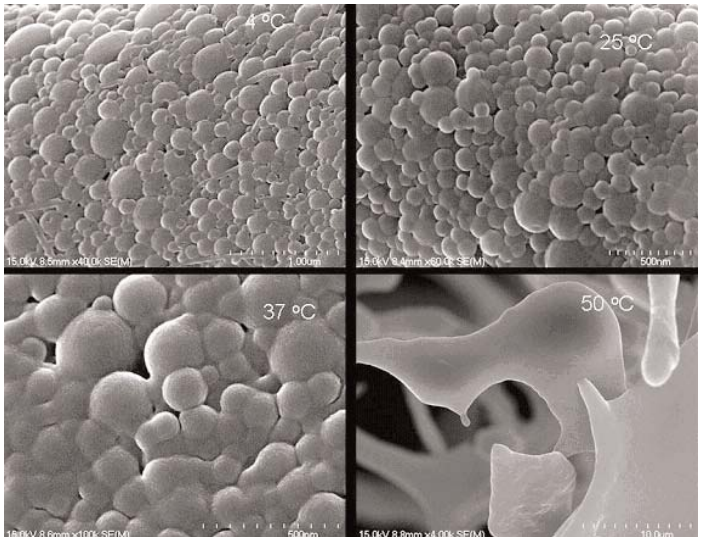

Figure 7. SEM photomicrographs illustrating the effect of temperature of storage on the extent of aggregation of 266-nm paclitaxel-PLGA nanospheres after storing at 4°C, 25°C, 37°C, and 50°C for 6 days.

tures, whereas at 50°C all residual solvent evaporated during storage. Further, the DSC was insensitive to detect any heat changes in the PLGA. The DSC profile of control, placebo PLGA nanospheres (4°C), PVA, and Bodipy were similar to the nanospheres containing Bodipy stored at elevated temperatures (Figure 6).

The aggregation of the particles during storage was independent of the entrapped Bodipy as similar temperature and size-dependent aggregation was observed with PLGA particles containing the nonionic, hydrophobic, anticancer drug, paclitaxel (Figure 7). In addition, SEM studies revealed that the extent of aggregation was similar for paclitaxel nanospheres as the storage temperature increased from 4°C to 50°C. Further, aggregation of microspheres during storage also occurred with samples prepared with PLGA 50:50 (intrinsic viscosity: 1.0 dL/g, Figure 8), PLA (intrinsic viscosity: 1.04 dL/g, Figure 9A), and PLGA 75:25 (intrinsic viscosity: 0.69 dL/g, Figure 9B). Importantly however, the extent of aggregation decreased as the lactic acid content of the copolymer increased. Further, increasing the molecular weight of PLGA 50:50 from 0.69 to 1.04 dL/g (~60 000 to 90 000 g/mol) did not significantly alter the extent of aggregation. It is unlikely that the compound, Bodipy, contributed to the aggregation because (1) the concentration in the particles was less than 0.03% wt/wt, and (2) similar aggregation occurred in paclitaxel-PLGA particles. It would be ideal to quantify the amount of residual dichloromethane in the PLGA nanospheres using sealed cap,

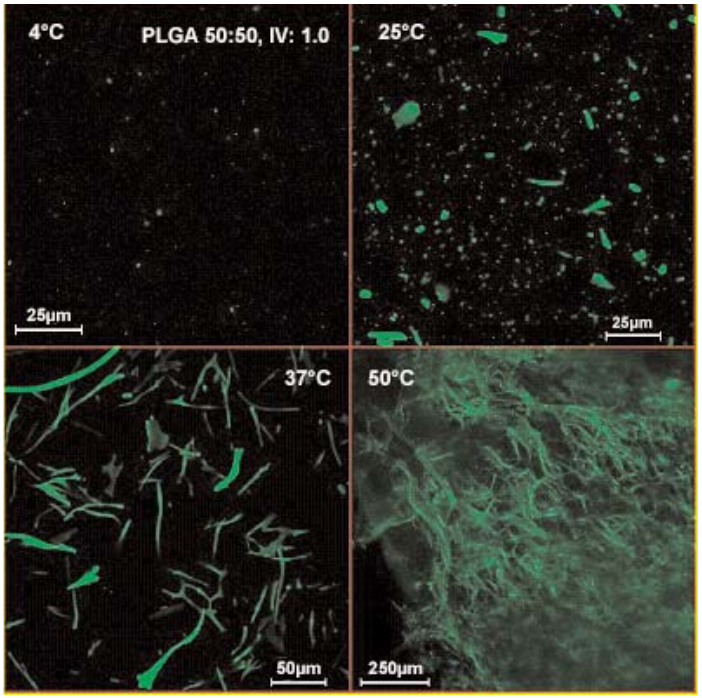

Figure 8. Effect of storage temperature on the extent of aggregation of 266-nm PLGA 50:50 (IV: 1.00 dL/g) nanospheres containing Bodipy, after storing at 4°C, 25°C, 37°C, and 50°C for 6 days.

head space analysis using gas chromatography-mass spectrometry (GC-MS). However, we believe this would be extremely difficult owing to (1) small sample size, (2) low concentration of dichloromethane, and (3) difficulty in extracting residual dichloromethane from within the nanospheres.

Effect of PVA Concentration and Residual Dichloromethane on the Extent of Aggregation

It is known that despite several washings, PVA forms a corona on the surface of the nanospheres.[22] Therefore, it is possible that the storage of the nanospheres at elevated temperatures may destabilize the PVA, causing aggregation. However, it was concluded that PVA did not cause aggregation of the nanospheres as the extent of aggregation was similar for the nanospheres prepared with varying PVA concentrations when stored at 4°C, 25°C, 37°C, and 50°C. Further, as the DSC and TGA thermograms stored at each temperature were almost identical, it was concluded that PVA was not responsible for aggregation.

The additional evaporation step was introduced to remove organic solvent during preparation of Bodipy-PLGA nanospheres (Figure 10). After lyophilization, the lyophilized nanospheres were compact, but redispersible. The time-dependent study of the extent of aggregation of the nanospheres subjected to the additional rotavap step was significantly less aggregated compared with the control samples after days 1 and 2 (Figure 10). This strongly suggests that during storage at higher temperatures, the residual dichloromethane (bp = 39.75°C) migrates to the surface of the nanospheres and dissolves the PLGA on the superficial layers causing coalescence of the nanospheres.

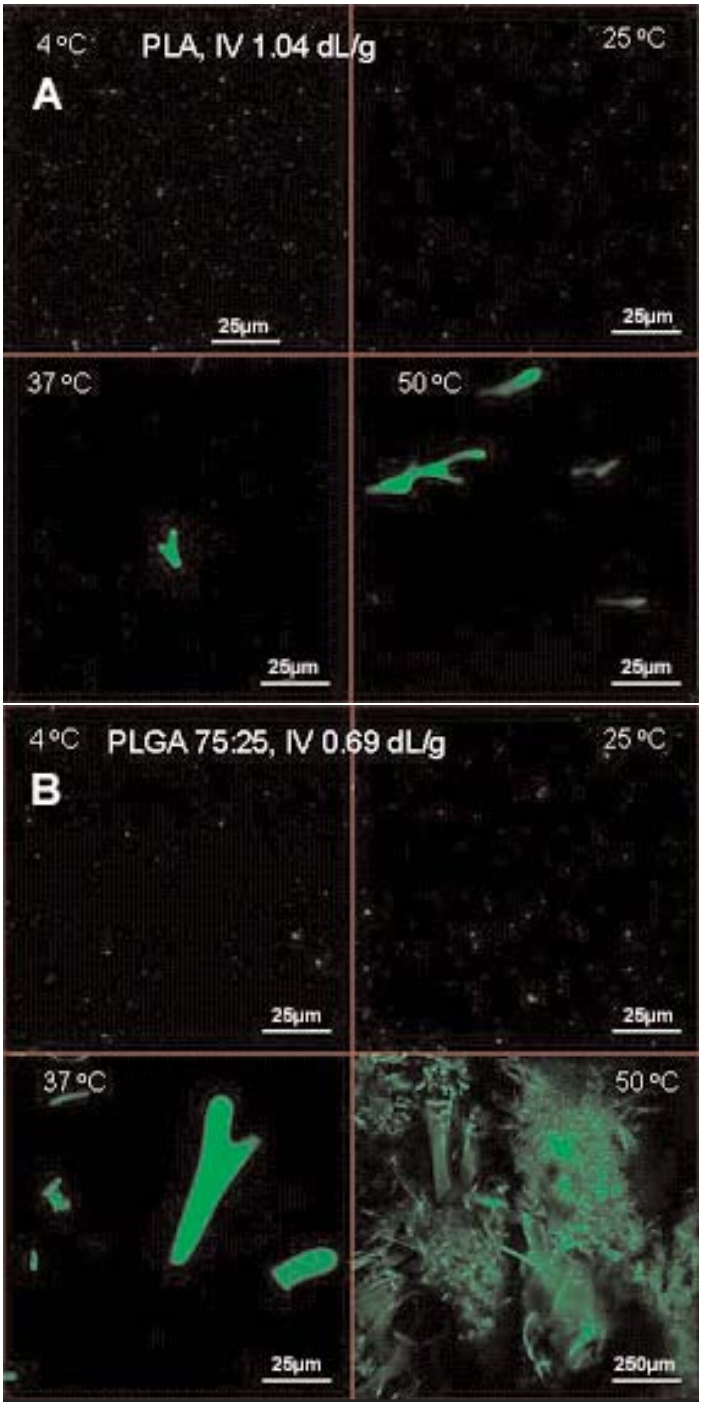

Figure 9. Effect of storage temperature on the extent of aggregation of 300-nm (A) PLA (IV: 1.04 dL/g) and (B) PLGA 75:25 (IV: 0.69 dL/g) nanospheres containing Bodipy, after storing at 4°C, 25°C, 37°C, and 50°C for 6 days.

CONCLUSION

To maintain the physical integrity of PLGA 50:50 nanospheres during storage, it is critical that they are stored at 4°C to avoid irreversible aggregation. The extent of coalescence of particles consistently increased as storage temperature

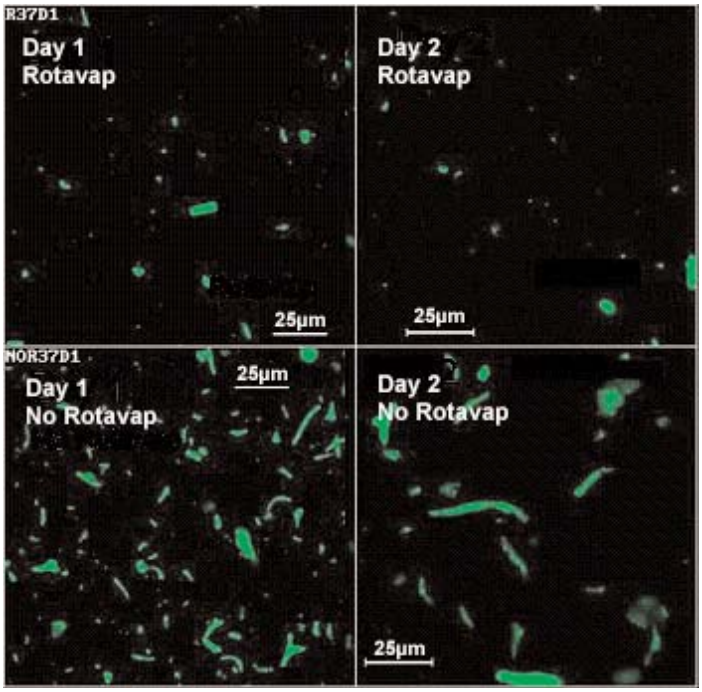

Figure 10. Reduction in aggregation of nanospheres after 1 and 2 days of storage at 37°C after rotary evaporation (rotavap) step was included during manufacture. Nanospheres with additional evaporation had minimal aggregation with temperature.

increased from 4°C to 50°C but decreased as the size of the particle increased from 300 to 2000 nm.

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