

Encapsulation of PROLI/NO in biodegradable microparticles

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Biodegradable hydrophilic polymers poly-lactic-co-glycolic acid (PLGA) and polyethylene oxide-co-lactic acid (PELA) were used to encapsulate a small hydrophilic prodrug (PROLI/NO) as a strategy to deliver nitric oxide (NO) by inhalation. The microparticles were prepared using double emulsion and solvent evaporation, followed by freeze-drying. The NO release kinetics were characterized by three parameters: the maximum concentration of NO per unit weight of microparticles, C_{\max} (nM mg^{-1}); the window of time for which the concentration exceeded 50% of C_{\max} , W_{50} (min); and the initial rate of release, R_i ($\text{nM mg}^{-1} \text{min}^{-1}$). PLGA-based microparticles did not encapsulate PROLI/NO. PELA-based microparticles demonstrated an entrapment efficiency rate of 43%, a mass median diameter of $2.3 \mu\text{m}$, and NO release in a physiological buffer characterized by $C_{\max} = 123$, $W_{50} = 4.11$, and $R_i = 78.7$. Addition of gelatin as a hydrophilic binding moiety in the first emulsion allowed PLGA-based microparticles to encapsulate PROLI/NO; however, the mass median diameter was too large for inhalation ($23.5 \mu\text{m}$). It is concluded that the hydrophilic polyethylene glycol-moiety in PELA allows for efficient encapsulation of PROLI/NO, and PELA-based microparticles might be a strategy to generate a stable inhalable form of NO.

Keywords: Pulmonary hypertension, inhalation, nitric oxide, PELA, PLGA, microsphere.

Introduction

Nitric oxide (NO) is a potent vasodilator and has been used to relieve pulmonary hypertension and acute respiratory distress syndrome (ARDS) by inhalation in the gas phase (Rubanyi 1991, Zapol and Hurtford 1994, Moncada and Higgs 1995). As a gas, NO is effectively delivered to the smooth muscle of pulmonary arteries, and excess NO is quickly scavenged by substrates such as haemoglobin in the circulating blood such that systemic hypotension may be prevented. However, the clinical effect is short-lived ($< 1\text{--}2$ min), and thus gas phase delivery requires patients to wear a nasal cannula continuously and to carry cumbersome equipment such as a compressed gas tank and regulator.

Inhalation of NO-releasing prodrugs provides a potential alternative (Mooradian *et al.* 1995, Keefer 1998). NONOates (diazonium diolates) such as DEA/NO, DETA/NO or DPTA/NO have been reported to release NO in solution (Keefer

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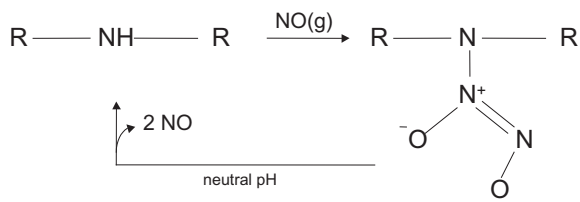


Figure 1. Formation of diazenium diolates (NONOate) and NO release under physiological pH conditions. R, alkyl or aminoalkyl side group.

et al. 1996) and are easily synthesized from the corresponding secondary amines, DEA, DETA and DPTA (figure 1). NONOates have a range of NO-releasing kinetics and could potentially be inhaled as an aqueous aerosol to target the deep lung or alveolar region. However, most secondary amines are mutagenic and thus pose long-term health risks for a chronic disease such as pulmonary hypertension (Mirvish *et al.* 1980). One exception is the amino acid proline (Keefer 1998), which can be used to synthesize PROLI/NO. However, PROLI/NO releases NO very quickly at a neutral pH (half-life = 1.8 s) (Saavedra *et al.* 1996). Thus, it cannot be effectively stored or delivered in a neutral aqueous aerosol phase, and its potential clinical effect would be short-lived.

This study presents a strategy for an inhalable, biodegradable microparticle system to deliver PROLI/NO to the alveolar region of the lungs. The delivery system must have the following characteristics: (1) 1–3 μm diameter particles (Edwards *et al.* 1997, 1998), (2) stable at room temperature, (3) slow release of NO and (4) biodegradable. Many biodegradable polymers are appropriate for making microparticles (Edwards *et al.* 1997, Deng *et al.* 1999, 2001, Kawashima *et al.* 1999) to entrap large molecules such as peptides or vaccines. PLGA (poly-lactic-co-glycolic acid) and PELA (polyethylene oxide-co-lactic acid) were chosen as candidate polymers due to their ability to biodegrade (Deng *et al.* 2001), to form microparticles, and potentially to entrap a small hydrophilic prodrug such as PROLI/NO.

Materials and methods

Prodrug (PROLI/NO) synthesis

PROLI/NO was synthesized as reported by Saavedra *et al.* (1996). Briefly, 10 g L-proline were solubilized in 39 ml 25% sodium methoxide in methanol (all Aldrich Chemical Co., Milwaukee, WI, USA). After degassing with Ar (Airgas, Radnor, PA, USA), the solution was sealed in a pressure bottle (Ace Glass, Inc., Vineland, NJ, USA), and NO gas (Airgas) was supplied to the headspace of the solution for 12 h maintaining the pressure at 50 psig while stirring. The pressure was released and the solution precipitated with 3 vols ether (Aldrich). The precipitates were recovered by filtration, washed with 1 vol. ether and dried under vacuum overnight. The white powder product was identified as PROLI/NO by ultraviolet light spectrophotometry at 251 nm after dissolving in 100 mM NaOH solution. In addition, the compound released NO by diluting in Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, Inc., St Louis, MO, USA)

and measuring the subsequent increase in NO concentration (inNO-T with amiNO-700 probe; Innovative Instruments, Inc., Tampa, FL, USA). The dried compound was stored at -80°C , and small aliquots were stored at -20°C for short-term storage before use.

Polymers

PLGA (5050 DL, acid end group) was donated by Alkemes, Inc. (Cincinnati, OH, USA). PELA was synthesized from polyethylene glycol (PEG, MW 4600, Aldrich) and d,l-lactide (Polysciences, Inc., Warrington, PA, USA) (Deng *et al.* 1990). Two different contents of PEG (5 and 10% by weight) were used. Briefly, PEG was dried in a vacuum oven at 45°C for 2 days, and then combined with d,l-lactide in a round-bottomed flask. After applying vacuum and Ar gas alternately, the flask was heated to 180°C in a silicone oil bath. After the reactants melted, $3.6\ \mu\text{l}$ stannous 2-ethyl hexanoate (Sigma Chemical Co., St Louis, MO, USA) was added as a catalyst, and polymerization was allowed to proceed for 8 h. The resulting polymer was cooled and dissolved in acetone ($5\ \text{ml g}^{-1}$ polymer) overnight. The polymer solution was then added to 200 ml distilled water and recovered as the precipitate after centrifugation and filtration. The polymer was dried overnight under vacuum, and the molecular weight was determined by SEC-HPLC column (PLgel 5 micron column, Polymer Laboratories, Inc., Amherst, MA, USA) and system (LC-10AT HPLC system, Shimadzu Co., Tokyo, Japan) using THF (Aldrich) as a mobile phase and polystyrenes (Aldrich) as molecular weight standards.

Microparticle preparation and drug encapsulation

Microparticles were prepared by double emulsion. A total of 10–200 mg PROLI/NO were dissolved in 0.5–1 ml 100 mM NaOH solution or 5% gelatin solution (pH 11), and then mixed with 5 or 10% polymer solution in methylene chloride (Aldrich). The combined solution was instantly homogenized (PowerGen 700, Fisher Scientific, Pittsburgh, PA, USA) briefly (2–5 s) to create the water in oil (w/o) emulsion. Following homogenization, the emulsion was introduced into different volumes of 1% poly(vinyl alcohol) (PVA; Polysciences) in water, then followed by a second homogenization for 1 min to create the w/o/w emulsion. After 30 min of stirring with a magnetic stirrer to evaporate the solvent, the emulsion was centrifuged, and the precipitates collected, and resuspended with a 10% lactose solution, and then freeze-dried (Labconco Co., Kansas City, MO, USA). The morphology of the microparticles was investigated by optical microscopy (Eclipse TE300, Nikon, Inc., Melville, NY, USA), and the size distribution determined by a particle size analyser (LS 100Q, Coulter Corp., Miami, FL, USA). Mass (volume) mean diameter (MMD) and standard deviation (SD) were based on the volume per cent of particles in the aqueous solution sent to the LS 100Q (total particles represented 10% of the aqueous volume).

Assay for mass balance of drug

To calculate the entrapment efficiency of PROLI/NO, nitrate (stable oxidized end-product of NO, and stoichiometrically equivalent to NO) was assayed by a

colorimetric Griess method (Sigma). The nitrate content of the supernatant during each step in the preparation of the microparticles was assayed to determine lost NO. Entrapped NO was then determined by subtracting the lost NO from the starting or initial NO (subtraction technique). To validate the subtraction technique, a known mass of the microparticles was dissolved in methylene chloride, extracted with the same volume of distilled water, and the nitrate content of the subsequent aqueous phase measured to determine the encapsulated NO. As a typical case (PELA-based microparticle), 125 μmoles NO were initially added as the prodrug. The entrapment efficiency was then defined as the percentage of the initial NO that was entrapped in the microparticles. A series of experiments ($n=3$) demonstrated that the amount encapsulated by subtraction (total NO minus amount lost in supernatant, $64.5 \pm 5.0 \mu\text{moles}$) was not different than that obtained by dissolution of the microparticles ($67.0 \pm 9.3 \mu\text{moles}$). Thus, subsequent studies used only the subtraction technique to determine entrapment efficiency.

NO release kinetics

Characterizing the NO releasing pattern of the dried microparticles was determined by placing 10–40 mg microparticles into a neutral pH solution in a 20-ml closed vessel without head space. The solution was stirred with a magnetic bar and the NO concentration profile was followed in time at a sampling rate of 30 Hz (inNO-T, Innovative Instruments). For comparison, a small bolus of alkaline solution of PROLI/NO was added before monitoring of the microparticle sample. NO concentration profiles were characterized by three indices: (1) the maximum concentration of NO per unit weight of microparticles, C_{max} (nM mg^{-1}); (2) the window of time for which the concentration exceeded 50% of C_{max} , W_{50} (min); and (3) the initial rate of release, R_i ($\text{nM mg}^{-1} \text{min}^{-1}$). R_i was determined using linear least-squares regression between time zero and the time at which C_{max} occurred. Choosing earlier time points than the time at C_{max} did not significantly impact the estimation of R_i . C_{max} , R_i and W_{50} are shown in figure 2.

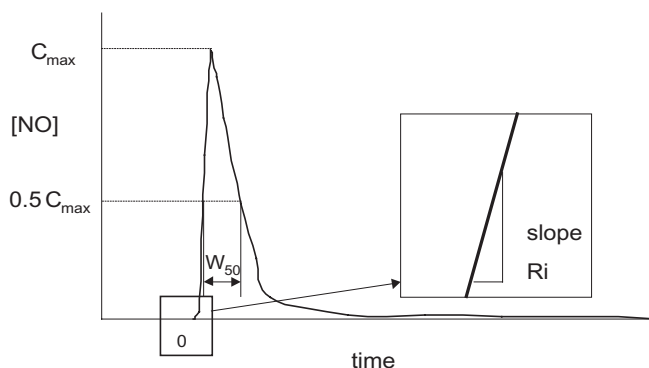


Figure 2. Schematic of the indices characterizing NO release kinetics: peak concentration (C_{max}), window of time the concentration exceeds 50% of the maximum (W_{50}), and initial release rate (R_i).

Results

PROLI/NO synthesis and NO release

PROLI/NO was successfully synthesized with a one-step reaction with NO gas under a high pressure. The identity of the product was determined by UV spectroscopy (251 nm) in NaOH solution where the compound is stable (Saavedra *et al.* 1996). NO release from PROLI/NO was verified with the colorimetric Griess assay, as well as direct measurement with an NO measuring probe and on-line measuring system (inNO-T), after diluting the alkaline PROLI/NO solution into a physiological buffer solution (20 mM DPBS). The on-line measurement demonstrated a near instantaneous peak of NO concentration after adding alkaline PROLI/NO to the buffer, followed by a decrease in concentration due to oxidation. A representative profile is shown in figure 5A. Means ($n = 3$) for W_{50} and R_i were 4.23 min and $142 \text{ nM mg}^{-1} \text{ min}^{-1}$, respectively, ($n = 3$).

Characterization of PELA

Synthesized PELA was analysed with HPLC to determine the molecular weight (MW) of the polymer. The range of MW was 10 000–20 000 under various conditions of synthesis and recovery. The expected functional groups in the polymer chain were identified with NMR analysis. PEG content (5 or 10%) did not affect the MW.

Characterization of microparticles

Figure 3 shows the microparticles made from PELA and PLGA by the double emulsion/solvent evaporation technique. Both microparticles have regular spherical morphology. Most PELA-based microparticles (PEG content of 5 or 10%) demonstrate a hollow central region (inner sphere) (figure 3A), but PLGA-based

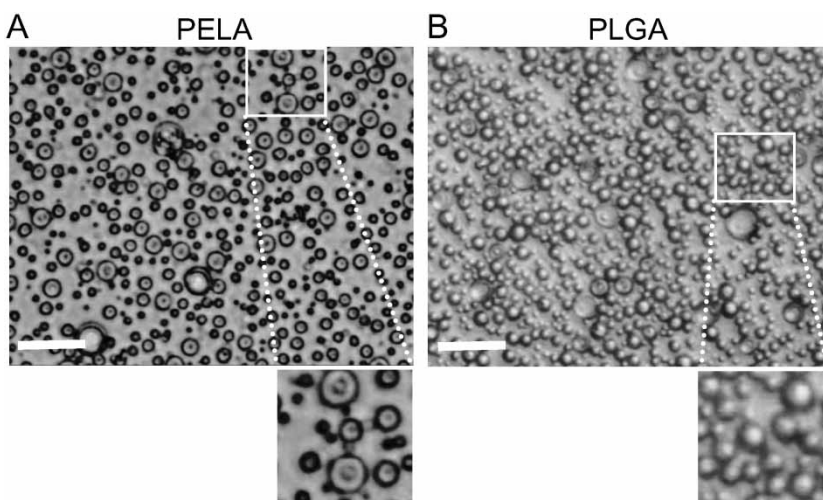


Figure 3. Bright-field microscopy of synthesized microparticles. (A) PELA-based (5% PEG); (B) PLGA-based. The white box highlights the region shown at higher magnification in the lower panel. Solid white bar = 10 μm .

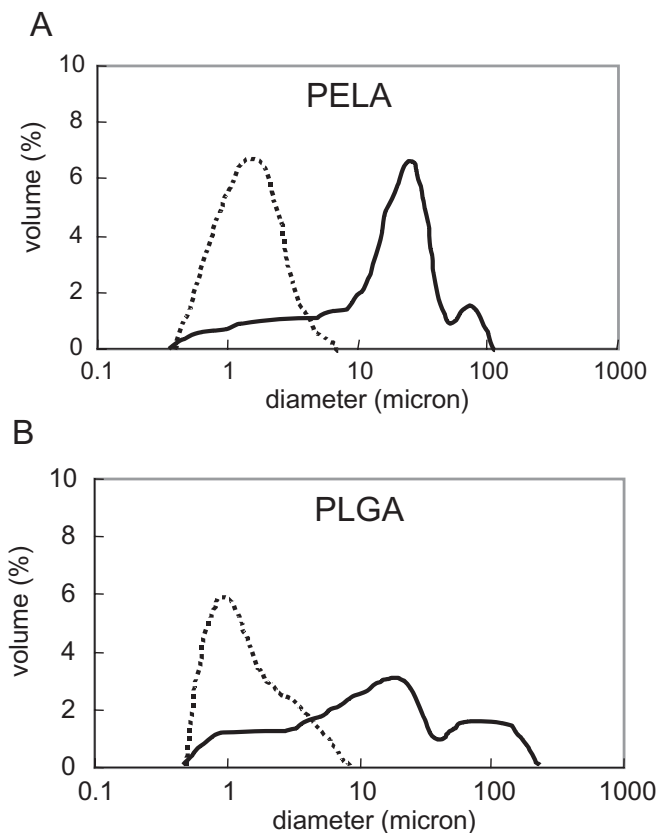


Figure 4. Size distribution profile of microparticles containing PROLI/NO in the presence (solid line) and absence (dashed line) of gelatin. (A) PELA-based (5% PEG); (B) PLGA-based.

microparticles are more homogeneous in morphology (figure 3B). For PELA-based microparticles, the ratio of the second emulsion volume to the oil phase volume was important in generating discrete microparticles of desirable size. The optimal ratio was 5 (20 ml/4 ml). At a larger ratio of 20 (80 ml/4 ml), non-discrete larger particles having multiple vesicles were formed (data not shown).

Figure 4 demonstrates the size distribution of PELA-based (A) and PLGA-based (B) microparticles having spherical morphology. The volume per cent of each size is plotted (dashed lines) on log scale of particle size. Table 1 shows the variances in the MMD and standard deviations for each case. For PLGA-based microparticles, the mean size was slightly smaller than PELA-based microparticles, but the distribution pattern and variances were similar (table 1).

NO encapsulation and release

Table 1 shows the entrapment efficiencies of the preparations. PELA-based microparticles demonstrated an entrapment efficiency of 43% (PELA without gelatin). In contrast, PLGA-based microparticles did not entrap PROLI/NO (PLGA without gelatin).

Table 1. Nitric oxide release characteristics and size distributions of microparticles.

| <i>n</i> | PELA (5% PEG) | | PLGA | |
|---|-----------------|--------------|-----------------|--------------|
| | No gelatin 5 | Gelatin 2 | No gelatin 3 | Gelatin 2 |
| Entrapment efficiency (%) | 43 ± 8 | 54 ± 8 | 0 ± 5 | 46 ± 10 |
| Particle size | | | | |
| MMD (μm) | 2.3 ± 0.54 | 15.6 ± 5.6 | 1.9 ± 0.56 | 23.5 ± 9.5 |
| SD (μm) | 1.2 ± 0.09 | 19.4 ± 3.6 | 1.6 ± 0.33 | 31.0 ± 5.5 |
| NO release | | | | |
| C_{\max} (nM/mg ⁻¹) | 123 ± 7.6 | 175 ± 56.7 | < 1 | 106 ± 18.0 |
| W_{50} (min) | 4.11 ± 0.52 | 2.43 ± 1.23 | – | 4.45 ± 0.67 |
| R_i (nMmg ⁻¹ min ⁻¹) | 78.7 ± 2.1 | 334 ± 116 | – | 115 ± 3.7 |

Data are Mean ± SD.

Figure 5 (B, C) shows the NO release profile from PELA-based (5% PEG) and PLGA-based microparticles, respectively. After adding the microparticles, NO is released from the prodrug and subsequently oxidized. The concentration is normalized by the weight of microparticles added. For the case of PLGA-based microparticles, NO was not released. For PELA-based microparticles, NO was released ($R_i = 78.7$), reaching a maximum concentration of 123 nM mg⁻¹ (C_{\max}), at a slower rate than PROLI/NO alone (figure 5A). The concentration remained above 50% of the maximum for 4.11 min (W_{50}). Increasing PEG content to 10% did not significantly impact NO release.

Effect of gelatin

To improve potentially the entrapment efficiency and gain insight into observed differences between PELA- and PLGA-based microparticles, gelatin was added in the single emulsion as a hydrophilic binding molecule for the prodrug. The entrapment efficiency increased in both polymer matrices, but PLGA-based microparticles showed a much more dramatic increase (table 1). Figure 5 (D, E) shows NO release from the microparticles prepared with gelatin. The PELA-based microparticles demonstrated a higher C_{\max} , faster initial NO release (R_i), and narrower time of release (small W_{50}) compared with the preparation without gelatin (table 1). The PLGA-based microparticles demonstrated a dramatic increase in NO release similar to the NO release profile of PELA-based microparticles without gelatin (figure 5B).

Adding gelatin (figure 4 and table 1) altered the size distributions for both PELA- and PLGA-based microparticles. The mean diameters dramatically increased and the width of the distributions (standard deviation) also increased. Microscopy also revealed large microparticles that were inhomogeneous in shape and multivesicular in appearance.

Discussion

A drug delivery system designed for the delivery of NO by inhalation has been developed. The small hydrophilic prodrug molecule, PROLI/NO, was

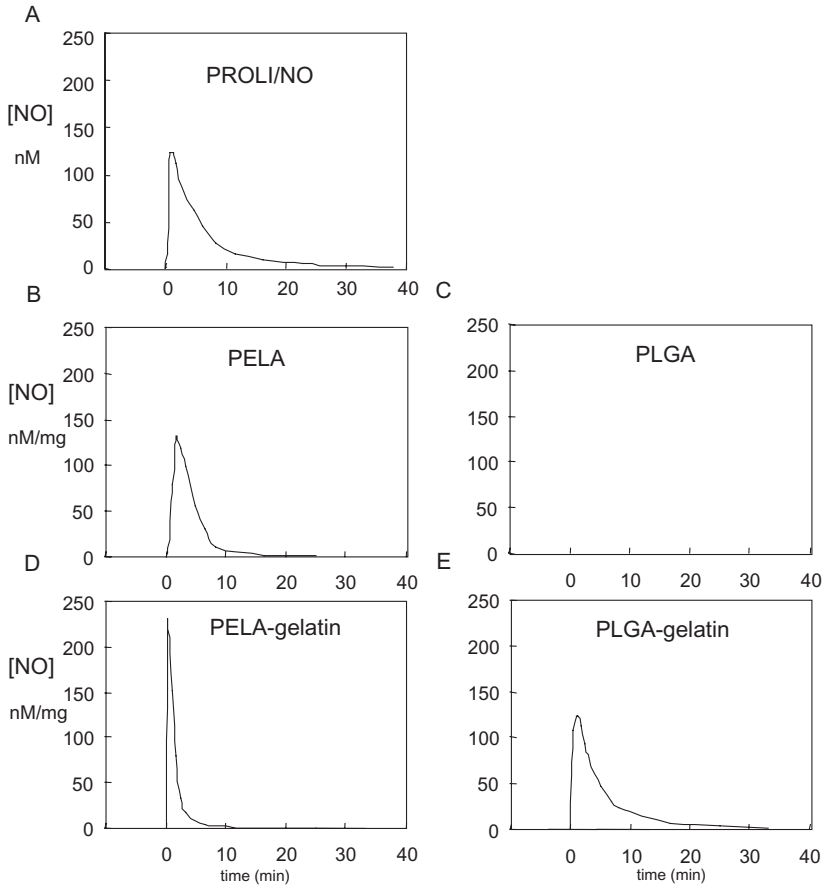


Figure 5. NO release kinetics monitored directly in a physiological buffer (DPBS) following the addition of a small volume of PROLI/NO in solution alone, or dried PROLI/NO-containing microparticles. (A) PROLI/NO alone; (B) PELA-based (5% PEG); (C) PLGA-based; (D) PELA-based with gelatin; (E) PLGA-based with gelatin.

encapsulated in a biodegradable polymer matrix (PELA or PLGA) to generate stable microparticles for inhalation. PLGA-based microparticles did not encapsulate PROLI/NO, and thus did not release NO. In contrast, PELA-based microparticles successfully encapsulated NO in microparticles that have a reasonable size distribution for inhalation, and demonstrated NO release in a physiologic aqueous solution.

As candidate degradable polymers, a well-known biodegradable polymer, PLGA, and a relatively new biodegradable polymer, PELA, were used as both polymers have been reported to be successful for encapsulating drug molecules. The drug molecules previously reported were large peptide or protein molecules (Deng *et al.* 2001) or hydrophobic small molecules (Dalpiaz *et al.* 2001), which are relatively easily entrapped in polymer matrices. PLGA is one of the more hydrophilic biodegradable polymers, but PELA is expected to be even more hydrophilic than PLGA due to the presence of a PEG moiety that has an extremely high affinity to water. PELA has been reported to have a high

entrapment yield when used for encapsulating hydrophilic protein molecules (Deng *et al.* 1999, 2001).

Previous results suggested that the relative hydrophilicity of PROLI/NO was large enough that a traditional single emulsification would not be a feasible entrapment strategy. Hence, a double emulsion technique (Alex and Bodmeier 1990, Maa and Hsu 1996, Maa and Hsu 1997) was used to entrap the whole aqueous phase of the drug in the polymer matrix. The present results demonstrated that only PELA (and not PLGA) could entrap PROLI/NO and subsequently release NO when the microparticles were soaked in a physiological buffer solution. A shorter time of solvent evaporation (30 minutes) was used because the prodrug is unstable in the aqueous phase at neutral pH, and the molecule diffuses rapidly due to its small size. This was possible because the later freeze-drying step was effective in removing the remaining solvent.

In so far as the particle density is similar to water, the particle size distribution demonstrates that the PELA- and PLGA-based microparticles are reasonable for inhalation and deposition in the alveolar region (diameter 1–3 μm). If the density of the particles is substantially different from water, then the size distribution can be adjusted by altering the characteristics of the homogenization steps during particle synthesis. The primary objective of the present study was to establish the feasibility of encapsulating an NO donor in a biodegradable and stable form. Optimizing the aerodynamic diameter for inhalation would need to be addressed in future experiments.

Although the PEG content was doubled to 10% in the PELA-based microparticles, the entrapment efficiency rate varied at < 5%. PELA-based microparticles demonstrated a central hollow space (hollow inner sphere), which represents the volume occupied by the PROLI/NO-rich aqueous phase before freeze-drying. In contrast, PLGA-based microparticles showed a compact surface and homogeneous interior suggesting the relatively hydrophobic polymer phase expelled the PROLI/NO-rich aqueous phase in the second emulsification. This might explain the lack of NO release by the PLGA-based microparticles and low entrapment yield (table 1).

From the success of adding the PEG moiety (increasing hydrophilicity) to the polymer matrix, addition of gelatin (hydrophilic binder) to the first aqueous drug phase was tried as another design concept. PLGA, which previously showed no entrapment without gelatin, showed very similar entrapment efficiency and release kinetics to those of the successful PELA-based microparticle (table 1 and figure 5). However, the particle size distribution was dramatically altered with particles that were too large for inhalation (figure 4).

Addition of gelatin impacted on not only the size distribution of both PELA- and PLGA-based microparticles, but also the NO release kinetics for PELA-based microparticles (figure 5D). This result provides insight on the mechanism of NO release from PROLI/NO in microparticles. In the presence of gelatin, the entrapment of PROLI/NO increased, but the rate of NO release also increased (increase in R_i and decrease in W_{50}). The enhanced hydrophilic moiety may draw water faster into the inner prodrug-containing space. Hence, the initial release rate of NO may be increased, and the NO release duration (W_{50}) might be decreased. The relative hydrophilicity of the microparticle core serves to enhance entrapment, but will also accelerate the rate of drug release.

A drug delivery system designed to deliver NO by inhalation using the encapsulation of the small hydrophilic prodrug PROLI/NO in the biodegradable polymer PELA has been presented. The microparticles are stable at room temperature and demonstrate a reasonable size distribution for inhalation and NO release. The rate of release was slower than PROLI/NO alone; however, even slower release rates may be attractive for routine clinical use and should be the focus of future studies. It is concluded that PELA-based microparticles are an effective means of stabilizing PROLI/NO as a strategy for pulmonary NO delivery and may also be useful for encapsulating other small hydrophilic drugs.

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References

- ALEX, R. and BODMEIER, R., 1990, Encapsulation of water-soluble drugs by a modified solvent evaporation method. I. Effect of process and formulation variables on drug entrapment. *Journal of Microencapsulation*, **7**, 347–355.
- DALPIAZ, A., SCATTURIN, A., PAVAN, B., BIONDI, C., VANDELLI, M. A. and FORNI, F., 2001, Poly(lactic acid) microspheres for the sustained release of a selective A1 receptor agonist. *Journal of Controlled Release*, **73**, 303–313.
- DENG, X., ZHOU, S., LI, X., ZHAO, J. and YUAN, M., 2001, *In vitro* degradation and release profiles for poly-dl-lactide-poly(ethylene glycol) microspheres containing human serum albumin. *Journal of Controlled Release*, **71**, 165–173.
- DENG, X. M., LI, X. H., YUAN, M. L., XIONG, C. D., HUANG, Z. T., JIA, W. X. and ZHANG, Y. H., 1999, Optimization of preparative conditions for poly-DL-lactide-polyethylene glycol microspheres with entrapped *Vibrio Cholera* antigen. *Journal of Controlled Release*, **58**, 123–131.
- DENG, X. M., XIONG, C. D., CHENG, L. M. and XU, R. P., 1990, Synthesis and characterization of block copolymers from D,L-lactide and poly(ethylene glycol) with stannous chloride. *Journal of Polymer Science*, **28**, 411–416.
- EDWARDS, D. A., BEN-JEBRIA, A. and LANGER, R., 1998, Recent advances in pulmonary drug delivery using large, porous inhaled particles. *Journal of Applied Physiology*, **84**, 379–385.
- EDWARDS, D. A., HANES, J., CAPONETTI, G., HIKACH, J., BEN-JEBRIA, A., LOTAN, N., ESKEW, M. L., ULTMAN, J. and LANGER, R., 1997, Large porous aerosols for pulmonary drug delivery. *Science*, **276**, 1868–1871.
- KAWASHIMA, Y., YAMAMOTO, H., TAKEUCHI, H., FUJIOKA, S. and HINO, T., 1999, Pulmonary delivery of insulin with nebulized DL-lactide/glycolide copolymer (PLGA) nanospheres to prolong hypoglycemic effect. *Journal of Controlled Release*, **62**, 279–287.
- KEEFER, L. K., 1998, Nitric oxide-releasing compounds: from basic research to promising drugs. *Chemtech*, **28**, 30–35.
- KEEFER, L. K., RAYMOND, W., DAVIES, K. M. and WINK, D. A., 1996, 'NONOates' (1-substituted diazen-1-ium-1,2-diolates) as nitric oxide donors: convenient nitric oxide dosage forms. In *Methods in Enzymology* (Washington: Academic Press). pp. 281–293.
- MAA, Y.-F. and HSU, C., 1996, Liquid-liquid emulsification by rotor/stator homogenization. *Journal of Controlled Release*, **38**, 219–228.

- MAA, Y.-F. and HSU, C. C., 1997, Effect of primary emulsions on microsphere size and protein-loading in the double emulsion process. *Journal of Microencapsulation*, **14**, 225–241.
- MIRVISH, S. S., BULAY, O., RUNGE, R. G. and PATLI, K., 1980, Study of the carcinogenicity of large doses of dimethylnitramine, N-nitroso-L-proline, and sodium nitrite administered in drinking water to rats. *Journal of the National Cancer Institute*, **64**, 1435–1442.
- MONCADA, S. and HIGGS, E. A., 1995, Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB Journal*, **9**, 1319–1330.
- MOORADIAN, D. L., HUTSELL, T. C. and KEEFER, L. K., 1995, Nitric oxide (NO) donor molecules: effect of NO release rate on vascular smooth muscle cell proliferation *in vitro*. *Journal of Cardiovascular Pharmacology*, **25**, 674–678.
- RUBANYI, G. M., 1991, Endothelium-derived relaxing and contracting factors. *Journal of Cellular Biochemistry*, **46**, 27–36.
- SAAVEDRA, J. E., SOUTHAN, G. J., DAVIES, K. M., LUNDELL, A., MARKOU, C., HANSON, S. R., ADRIE, C., HURFORD, W. E., ZAPOL, W. M. and KEEFER, L. K., 1996, Localizing antithrombotic and vasodilatory activity with a novel, ultrafast nitric oxide donor. *Journal of Medical Chemistry*, **39**, 4361–4365.
- ZAPOL, W. M. and HURTFORD, W. E., 1994, Inhaled nitric oxide in adult respiratory distress syndrome and other lung diseases. *Advances in Pharmacology*, **31**, 513–530.