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Combinatorial co-encapsulation of hydrophobic molecules in poly(lactide-co-glycolide) microparticles

Abhinav P. Acharya, Jamal S. Lewis, Benjamin G. Keselowsky*

J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, 130 BME/PO Box 116131, Gainesville, FL 32611-6131, USA

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ABSTRACT

There is great interest for developing poly(lactide-co-glycolide) (PLGA) based particles for targeted delivery and controlled release of encapsulated biological molecules. These PLGA particles can be used to deliver proteins, small molecule drugs and nucleotides. Furthermore, it has been shown that the co-encapsulation of multiple factors in PLGA particles can generate synergistic responses, and can also provide theranostic capability. However, the number of possible unique particle formulations that may be generated by the combination of different components in a particle increases dramatically with each new component, and currently, there is no method to generate large libraries of unique PLGA particles. In order to address this gap, we have developed a high-throughput methodology to produce hundreds of small batches of particles. The particles are generated in multi-well plate wells by a modified oil-in-water emulsion technique. In order to demonstrate the versatility of this technique, combinatorial formulations of six different loading concentrations of three fluorescent dyes were fabricated giving rise to 216 unique PLGA particle formulations. We demonstrate systematic and well-controlled combinatorial loading of hydrophobic molecules into the particles. This parallel particle production (PPP) methodology potentiates the generation of hundreds of different combinatorial particle formulations with multiple co-encapsulates in less than 24 h in standard polystyrene multi-well plates, thus providing rapid, low cost, high-throughput production. We envision that such a PPP library of particles encapsulating combinations of drugs and imaging modalities can subsequently be tested on small populations of cells in a high-throughput fashion, and represents a step toward personalized medicine.

1. Introduction

Use of the polymer, poly lactide-co-glycolide (PLGA), has had a large impact on contemporary biomedicine. PLGA has been approved by the U.S. Food and Drug Administration (FDA) for biodegradable surgical sutures and drug delivery products and, is utilized extensively for drug delivery vehicles, tissue engineering supports and combination products in pre-clinical and clinical research [1–6]. PLGA can be fabricated into scaffolds or particles for delivery of a wide range of biologically active molecules [7–9]. Release rates of encapsulates can be controlled by manipulation of molecular weight and/or the lactide to glycolide ratio of the polymer. Sizes of PLGA particles have been varied from 50 nm to 100 μm for use in drug delivery via various techniques, including solvent extraction/evaporation, phase separation (coacervation) and spray-drying [10–12]. Among these, phase separation and spray drying techniques are harsh on the encapsulates, while solvent extraction utilizes large amounts of reagents and is labor intensive. Although solvent evaporation is the most extensively used technique to generate PLGA micro/nano particles, it can only produce particles in batches, one formulation at a time. Our approach utilizes a miniaturized, highly parallel solvent evaporation technique in a multiplexed configuration for parallel generation of large numbers of different formulations with combinations of multiple co-encapsulated agents. Furthermore, this method is readily scalable in terms of increasing the number of uniquely formulated batches, and leverages the use of relatively inexpensive standard contact pin printing miniarraying equipment for programmable dispensing of hydrophobic molecules into wells of a polystyrene multi-well plate. We hypothesize this PPP method will generate PLGA particles in small "micro-batches" in a semi-automated fashion, requiring limited reagents and enabling the generation of a large multi-component particle library, appropriate for testing on small cell populations in a high-throughput fashion.

* Corresponding author. Tel.: +1 352 273 5878; fax: +1 352 392 9791.
E-mail addresses: bgk@ufl.edu, bkeselowsky@bme.ufl.edu (B.G. Keselowsky).

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2. Materials and methods

2.1. Materials

A 50:50 polymer composition of poly(d,l lactide-co-glycolide) (PLGA) with inherent viscosity 0.55–0.75 dL/g in hexafluoroisopropanol, HFIP (Lactel, AL, USA) was used to generate particles. Poly-vinyl alcohol (PVA) (MW ~ 100,000 g/mol, Fisher Science, Rochester, NY, USA) was utilized as an emulsion stabilizer. Phosphate buffered saline (PBS) solution (Hyclone, UT, USA) was used as the aqueous phase to form the emulsions while propylene carbonate (PC) (Fisher Scientific, NJ, USA) was used as an organic solvent to dissolve PLGA polymer. Microparticles were generated using solid-oil-water emulsion technique. Fluorescent dyes, 7-diethylamino-4-methylcoumarin (coumarin: $\lambda_{ex} = 375$ nm and $\lambda_{em} = 445$ nm), 1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide (cyanine: $\lambda_{ex} = 648$ nm and $\lambda_{em} = 670$ nm) and rhodamine 6G (rhodamine: $\lambda_{ex} = 528$ nm and $\lambda_{em} = 550$ nm) were encapsulated in the particles as representative hydrophobic molecules. Dyes were chosen so as to have minimum overlap of absorption of one dye with the emission spectrums of another, in order to reduce the quenching effect. Polystyrene 384-well plates were used as generation chambers for the particles and a Calligrapher Miniarrayer (Bio-Rad) contact printer was used to transfer solutions into these particle generation chambers.

2.2. Parallel particle production (PPP)

A 384-well plate containing six different dilutions of the three fluorescent dyes dissolved in propylene carbonate (PC) was used as a source plate. Coumarin was dissolved in PC in concentrations of 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL; rhodamine was dissolved in PC in concentrations 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL and cyanine was dissolved in PC in concentrations of 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL. A 40 μL volume of each dilution was added to the wells of 384 well plates. Additionally, a single well was filled with 40 μL volume of PC without any dye to serve as the "zero dye" control. These six dilutions of the dyes were then printed in a 384 well plate using the contact printer to form a 6 × 6 × 6 matrix, with all possible combinations of the three available dyes which resulted in 216 different formulations at room temperature. The dye-printed plate was analyzed using a plate reader with appropriate filters ($\lambda_{ex}/\lambda_{em} = 375/445$; 648/670 and 528/550 nm) to measure fluorescence of the printed dyes to ensure accurate printing. After printing the dyes, 10 μL of 3% PLGA dissolved in PC was added to the wells using a multichannel pipette. The plate was then sonicated in a sonicating water bath for 3 min. Next 50 μL of 5% PVA solution in PBS was added to the wells and the plate was sonicated for 5 more minutes. The plate was then incubated for 16 h under a vacuum pressure of 0.2 mTorr at 37 °C to evaporate PC and water. A volume of 80 μL of de-ionized water was added to each well using a multichannel pipette and the plate was sonicated for 60 s. The plate was then centrifuged for 15 min at 1500 × G for 20 min. The supernatant was then removed and the particles re-suspended by sonicating in 50 μL/well of de-ionized water. This washing process of centrifuging, aspirating and re-suspending was repeated 5 times to remove PVA from the particles in the well. The plate was then frozen by placing over dry ice and dried under vacuum overnight. All the particle generation steps were performed in the dark to reduce photo-bleaching of the dyes.

2.3. Particle analysis

For analysis, the particles were re-suspended in de-ionized water solution using a sonicating bath and printed via Miniarrayer on a plasma cleaned glass microscope slides (Fisher Scientific, NJ, USA). Particle morphology was characterized by scanning electron microscope (FEG-SEM JEOL JSM – 6335F, Major Analytical Instrumentation Center, University of Florida). Particles from different micro-batches were combined, suspended in DI water and printed using a microarray printer. The array of particles was then dried for 16 h at room temperature. Dried particles were coated with 5–10 nm thickness of gold and imaged at magnifications ranging from 30× to 60,000×. Furthermore, particle formulations were combined from separate runs, to confirm the range of particle sizes obtained from SEM and particle size was analyzed by dynamic light scattering (volume estimation) using a Nanotrac (Microtrac Inc.) particle size analyzer. Flow cytometry (BD Biosciences) was utilized to analyze the range of fluorescence intensities of >10,000 particles within each microbatch. Flow

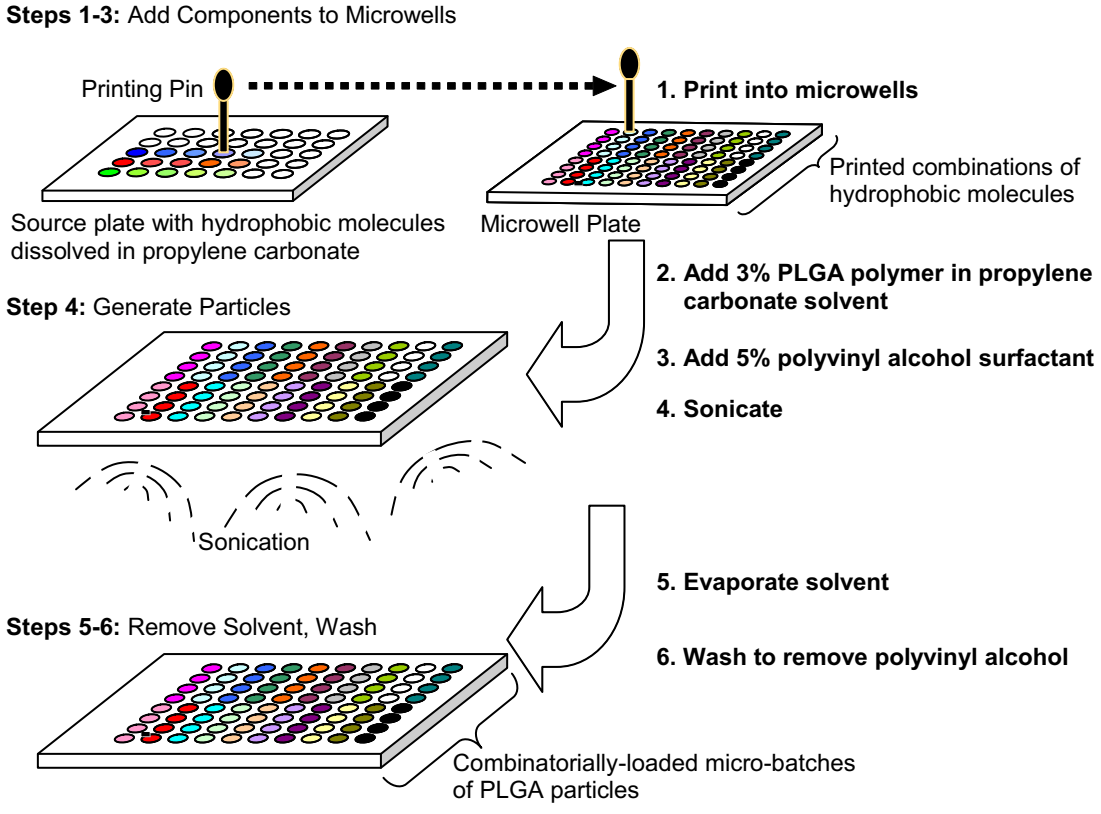

Fig. 1. Parallel particle production: Microparticles of poly(lactide-co-glycolide) (PLGA) co-encapsulating multiple hydrophobic components are generated using oil-in-water emulsification in a multi-well polystyrene plate serving as a bank of chambers in which hundreds of particle "micro-batches" are simultaneously generated. Fluorescent dyes are encapsulated as proof of principle, serving as a proxy combination drug formulation. The parallel particle production (PPP) method consists of: Step 1–3.) Dip-pen miniarrayer is programmed to combinatorially add multiple components to microwells: 1.) dilutions of fluorescent dyes in solvent propylene carbonate (PC) are prepared in the source plate and these solutions are then printed into wells of a microwell plate. 2.) PLGA dissolved in PC is added to the microwells and the mixture is sonicated to homogenize. 3.) Surfactant, polyvinyl alcohol (PVA) in water is added to microwells. Step 4.) generate Particles: Microwells, containing PVA, PLGA, PC and hydrophobic molecules (dyes) and are sonicated to form an emulsion. Step 5-6.) remove the solvent and wash: 5.) the microwell plate is placed in a vacuum chamber and the PC solvent is evaporated. 6.) Microparticles are washed by centrifugation and buffer exchange with water to remove PVA and residual PC.