Supplemental information

Evaluating Green Solvents for Sustainable PLGA Nanoparticle Synthesis

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Contents

Materials

Reagents

Poly(D,L-lactide-co-glycolide) (Resomer 502H, 7 kDa: 17 kDa, acid terminated), acetone, (-)-Ethyl l-lactate (EL), Cyrene(BioRenewable), γ-valerolactone (GVL)(BioRenewable, ≥99%, ReagentPlus®), 2-methyltetrahydrofuran (2-MeTHF)(BioRenewable, anhydrous, ≥99%, Inhibitor-free), Cyclopentyl Methyl Ether (CPME)(inhibitor-free, anhydrous, ≥99.9%), Dimethyl Isosorbide (DMI)(BioRenewable, ≥99%, ReagentPlus®), Dimethyl Carbonate (DMC) (anhydrous, ≥99%), n-(3-dimethylaminopropyl)-n′-ethylcarbodiimide hydrochloride (EDC HCl),N-Hydroxysuccinimide (NHS), HPLC grade acetonitrile, HPLC grade Dimethyl sulfoxide (DMSO), and Polyvinyl Alcohol (87-90% hydrolyzed, average mol. Wt. 30,000-70,00) were purchased from Sigma. Cyanine5 (Cy5) amine dye was purchased from Lumiprobe. 2% uranyl acetate (UA) solution was purchased from the UMN Characterization Facility.

1.5 mL Eppendorf tubes were purchased from Genesee Scientific. Borosilicate glass scintillation vials were purchased from VWR. 1cc 28Gx1/2" insulin syringes were purchased from Amazon. A D02-E100-05-N tangential flow filtration filter, ACTU-P13-25N PharmaPure #13 Tubing, and Spectra/Por dialysis membrane tubing (3.5 kDa MWCO) were purchased from Repligen. Polystyrene semi-micro cuvettes for the Malvern Zetasizer were purchased from Sarstedt and folded capillary cells were purchased from Fisher Scientific. Carbon coated mesh 400 copper grids were purchased from Electron Microscopy Sciences.

RPMI-1640 (ATCC), 1x PBS solution pH 7.4 (Corning), 1X Trypsin EDTA (Corning), Heat inactivated Fetal Bovine Serum (Gibco), Penicillin Streptomycin (Corning), and AlamarBlue HS Cell Viability Reagent (Invitrogen) were purchased from Fisher Scientific. Dulbecco's Modified Eagle Media (DMEM), Gelatin and Hydrocortisone were purchased from Sigma-Aldrich. Media 199 was purchased from GE Healthcare Life Science. Epidermal growth factor was purchased from PeproTech. Tissue culture plasticware was purchased from Genesee Scientific.

Cells

The OVCAR8 cells were a gift from the Hammond Lab. The LP9 cells were a gift from the Azarin lab. Cells were cultured at 37 °C with 5% CO_2. Cell line-specific culture information is provided in the 'Cell Culture', 'In Vitro Viability Studies' and 'In Vitro Cell Association studies' sections.

Methods

Dissolution of PLGA in each solvent

PLGA dissolution was tested at concentrations up to 20 mg/mL in each solvent. In most cases, vortexing was sufficient; however, in Cyrene, vigorous vortexing for 3 minutes (or until no visible polymer remained) was required to achieve complete dissolution. In contrast, PLGA did not dissolve in CPME or 2-MeTHF even after heating to 60 °C and shaking at 700 rpm in a thermal shaker.

Preparation of PVA solution

To prepare the PVA solution, the appropriate amount of PVA was weighed to achieve the desired concentration (1% or 2% w/v) and dissolved in milliQ water. The mixture was heated to 90 °C while stirring at 600 rpm in a 20 mL scintillation vial using a magnetic stir plate for 1 hour to allow complete dissolution of PVA.

PLGA Nanoparticle (NP) Synthesis Using Nanoprecipitation Technique

A previously established nanoprecipitation protocol[1] was used as the starting point for PLGA nanoparticle (NP) synthesis in nanoprecipitation-compatible solvents, including acetone, EL, Cyrene, Cyrene+2-MeTHF, GVL, and DMI. Briefly, PLGA was dissolved at 5 mg/mL in each solvent. 6 mL milliQ water added to a 20 mL scintillation vial under stirring at 500 rpm. 1 mL of PLGA-solvent solution was drawn into a syringe fitted with a 27-gauge needle. In the scintillation vial, the tip of the needle was submerged below the water line and the solution was then injected at a rate of 1 mL/min. The solvents were allowed to stir at 500 rpm for 15 minutes. After synthesis, the solution was further diluted with 8 mL milliQ water before purification.

PLGA NP Synthesis Using Solvent Emulsion Evaporation Technique

A previously described solvent emulsion evaporation technique was used with slight adjustments for synthesis of DCM and DMC-NPs as a starting point[2]. Briefly, 1% PVA solution was prepared in 5 mL milliQ water in a scintillation vial. PLGA was dissolved at a 5 mg/mL concentration in DCM or DMC. The PLGA-DCM or PLGA-DMC solution was pipetted into the 1% PVA solution, placed in an ice bath, and immediately subjected to probe sonication at 60% amplitude using a Cole Parmer Ultrasonic Processor for 5 minutes for DCM and 3 minutes for DMC. After synthesis, the solution volume was made up to 8 mL by adding milliQ water before purification.

PLGA-Cy5 Dye Conjugation

Stocks of Cy5 amine (30 mg/mL), EDC HCl (10 mg/mL), PLGA(10 mg/mL) , and NHS (10 mg/mL) were prepared in DMSO. EDC, at a stoichiometric molar excess of 1:5 (PLGA:EDC), and NHS, at a stoichiometric molar excess of 1:5 (PLGA:NHS), were mixed with PLGA in a glass vial and stirred with a magnetic stir bar for 3 hours, protected from light, to activate the carboxyl groups. To this mixture, Cy5 amine dye was added at a 1:2 (PLGA:Cy5 amine) molar ratio and stirred overnight.

The solution was then transferred to a 3.5 kDa MWCO dialysis membrane and dialyzed against 1:1 acetonitrile:water for 72 hours, with solvent changes every 4–6 hours.

Following dialysis, the solution was lyophilized using a Labconco Freezone Freeze Dry Systems Lyophilizer for 24 hours. Fluorescence of the PLGA-dye conjugate was quantified using a BioTek Synergy Hybrid H1 plate reader.

Synthesis of PLGA-Cy5 NPs

PLGA-Cy5 conjugate was dissolved in EL at a concentration of 25 mg/mL and PLGA was dissolved in 10 mg/mL in each of the green solvents. The PLGA-Cy5 conjugate was added at a 1:5 weight ratio (PLGA-Cy5:PLGA), and green solvents were added to achieve a final polymer concentration of 5 mg/mL. The synthesis protocols (Table 2) for each solvent were then used to produce the nanoparticles.

DLS Characterization of PLGA Nanoparticles

Dynamic light scattering (Malvern Zetasizer Pro, λ = 633 nm) was used to measure NP diameter and polydispersity (PDI). Zeta potential measurements were also obtained with the Zetasizer Pro using the laser doppler electrophoresis. NP solutions were diluted in milliQ water in polystyrene, semi-micro cuvettes for size measurements and DTS1070 folded capillary cuvettes for zeta measurements.

Centrifugation

To remove the solvents and surfactants after nanoparticle synthesis, 1 mL aliquots of NP solutions were placed in 1.5 mL centrifuge tubes and centrifuged at 12,000 rcf for 5 minutes at 24°C. This process was repeated for three complete washes with milliQ water.

Tangential Flow Filtration (TFF)

To remove solvents and surfactants after nanoparticle synthesis, the NP solution was placed in a 50 mL centrifuge tube and connected to a Repligen KrosFlo KR2i TFF system via Masterflex Teflon coated PharmaPure tubing. Purification of nanoparticles was carried out using a D02-E100-05-N membrane, with a 13 mL/min sample flow rate in a size 13 tubing, and milliQ water was used as a buffer. Each PLGA nanoparticle solution was washed until five diafiltration cycles were reached in the permeate. The sample was then recovered by reversing the flow and backflushing with 1 mL of millliQ water using the peristaltic pump. The particles were then concentrated and recovered using the same method. The NPs were then characterized using the DLS (see *DLS Characterization of PLGA Nanoparticles*).

TEM Imaging of PLGA Nanoparticles

A FEI Tecnai T12 TEM was used to image PLGA NPs. NPs were diluted to 0.25 mg/mL, and 10 µL of the diluted solution was deposited onto glow-discharged, carbon-coated mesh 400 copper grids. After 1 minute, the excess sample was removed by blotting. The grid was then stained with 10 µL of 2% uranyl acetate (UA) solution and immediately blotted. The UA staining process was repeated after 30 seconds and then the sample was left to dry for 10 minutes. A single-tilt sample holder was used to mount the grid. Particle morphology and size distribution were analyzed using a microscope operated at 120 kV with 10,000x–40,000x magnification. Bright-field images were recorded using a Gatan MSC794 CCD Camera.

Cell culture

OVCAR8 cells were maintained in RPMI-1640 media, supplemented with 10% (v/v) FBS and 1% (v/v) PS. LP9 cells were maintained in flasks pre-coated with gelatin (0.1 w/v%) in media prepared by mixing

1:1 ratio of Dulbecco's Modified Eagle Media (DMEM) and Media 199 supplemented with 15% (v/v) FBS, epidermal growth factor (EGF, 10 ng/mL), and hydrocortisone (0.4 μg/mL)

In Vitro Viability Studies

OVCAR8 cells were seeded at a density of 10,000 cells and LP9 cells at 15,000 cells/well in 90 μL culture medium in black 96-well bottom tissue culture treated plates and allowed to adhere for 24 hours. For each solvent, dosing concentrations of 0.025 mg/mL, 0.05 mg/mL, and 0.1 mg/mL were prepared in milliQ water for PLGA-NPs. 10 μL of each stock solution were added to the cells, followed by incubation for 24 hours or 48 hours. Cell media was removed after incubation and 100 μL of fresh media containing 10% alamarBlue by volume was added to each well, and cell viability was assessed after 1 hour of incubation. A BioTek Synergy Hybrid H1 plate reader was used to acquire the viability data.

In vitro Cell Association Studies

OVCAR8 cells were seeded in 24 well-clear bottom cell culture treated plates at a density of 100,000 cells per well in 225 μL media and allowed to adhere for 24 hours. Dosing stock of 0.1 mg/mL was prepared in milliQ water for each of the dye-conjugated PLGA-NPs. 25 μL of each stock was added to each well and incubated for 24 hours. After incubation, cells were washed once with 500 μL PBS in each well, and dissociated using 100 μL trypsin. Trypsin was quenched with 300 μL media and cells were then transferred to a fluorescence-activated cell sorting (FACS) tube through a cell strainer cap and placed on ice until analysis. For flow cytometry analysis, samples were analyzed using BD LSRFortessa-X20 cytometer. Samples were analyzed on the APC channel (ex. 640, filters 670/30). Data was analyzed using FlowJo software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism. Pairwise comparisons were conducted using the two-tailed unpaired t-test and Mann-Whitney test. Multiple comparisons were performed using Kruskal-Wallis test with post hoc Dunn's multiple comparisons test and one-way ANOVA with Tukey's multiple comparison test.

Supplemental table

Table S1. Effect of varying synthesis parameters on nanoparticles synthesized with Cyrene. The bolded entries indicate the specific changes made during each experiment.

Experiment no.	PVA concentration (%)	Sonication conditions	Stir conditions	Organic: Aqueous Ratio	Injection rate (mL/min)	Outcome
1	**0-5**	None	700 rpm, 3 hours	1:6	2	PLGA precipitated
2	1	**100%, 5 minutes**	700 rpm, 3 hours	1:6	2	Size: 350±1.1 nm
3	**2**	100%, 5 minutes	700 rpm, 3 hours	1:6	2	Size=267.4±0.5 nm
4	**3**	100%, 5 minutes	700 rpm, 3 hours	1:6	2	Size=288±1.6 nm
5	**2**	**100%, 10 minutes**	700 rpm, 3 hours	1:6	2	Size=278.0±3.2 nm
6	2	100%, 5 minutes	**500 rpm, 12 hours**	1:6	2	Size=270.7±2.2 nm
7	2	100%, 5 minutes	**None**	1:6	2	Size=266 ±4.2 nm
8	2	100%, 5 minutes	None	**1:3**	2	Size=310 ±6.6 nm
9	2	100%, 5 minutes	None	**1:8**	2	Size=325±3.7 nm
10	2	100%, 5 minutes	None	**1:6**	**0.5**	Size=225.7±2.2 nm

Supplemental figures

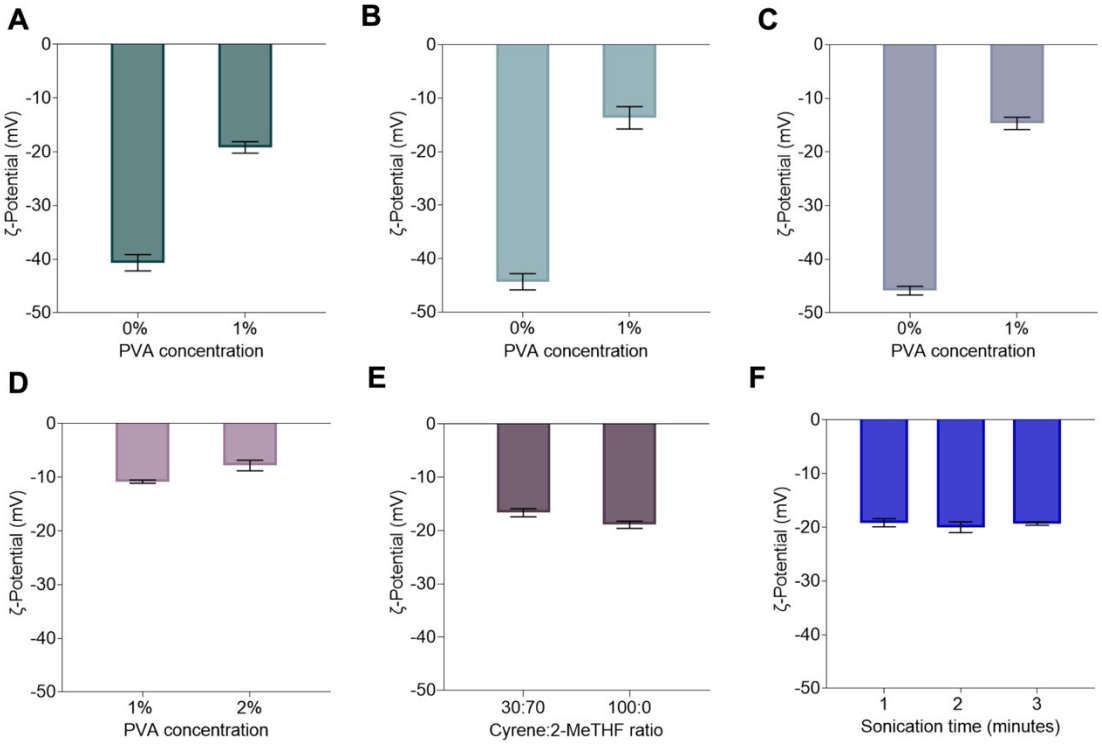

Figure S1. Effect of different synthesis parameters on zeta potential of (A) Acetone-NPs, (B) EL-NPs, (C) GVL-NPs, (D) DMI-NPs, (E) Cyrene-NPs, and (F) DMC NPs. Zeta potential was measured using Electrophoretic Light Scattering (ELS). Data are presented as mean ± standard deviation of three technical replicates.

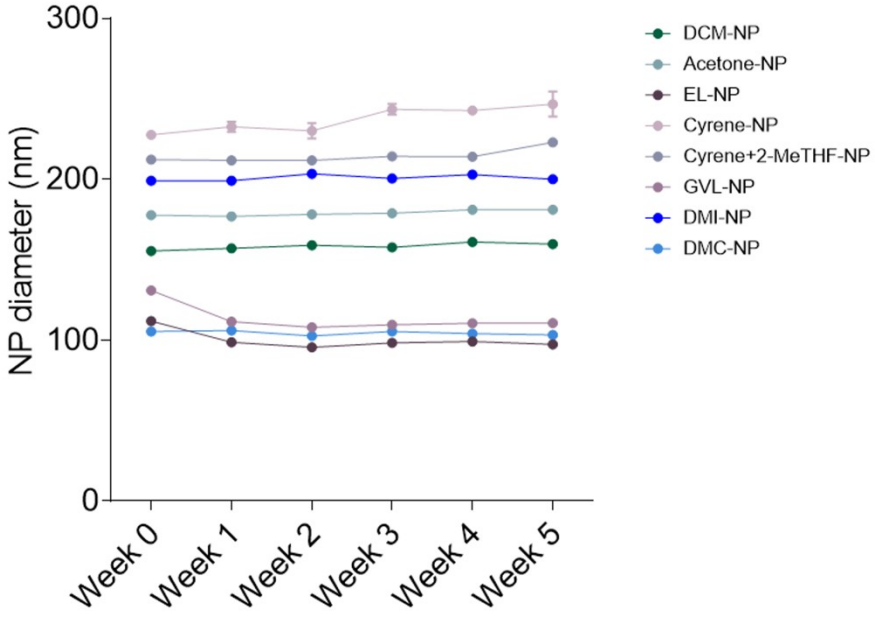

Figure S2. Stability of green solvent-NPs over 5 weeks. DLS characterization was performed to obtain NP diameter. Data are presented as mean ± standard deviation of three technical replicates.

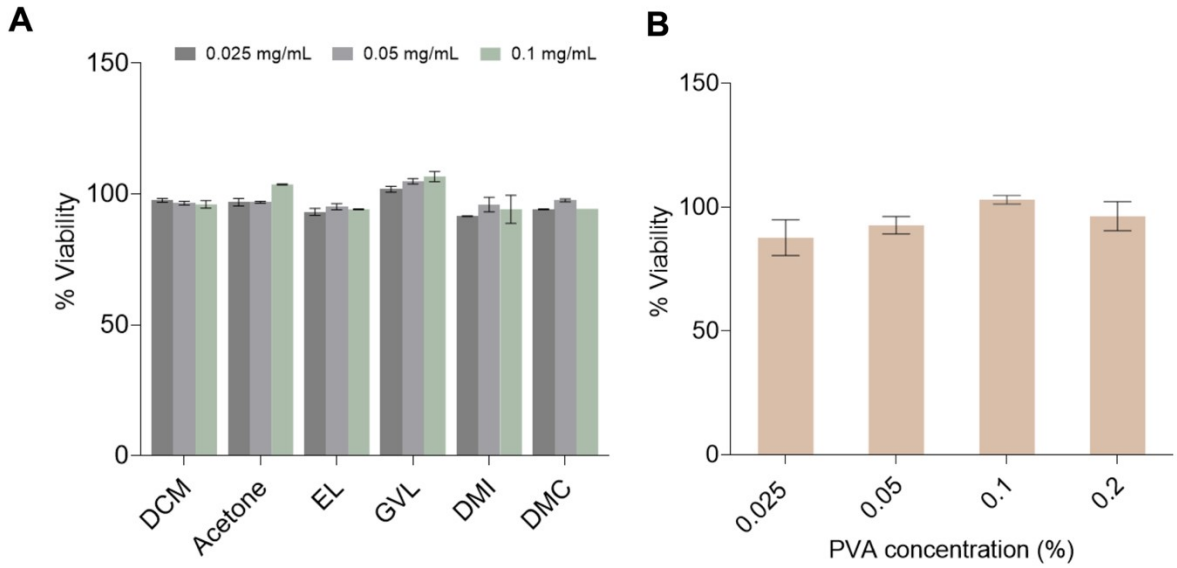

Figure S3. Cytotoxicity assessment of solvent and PVA only controls in OVCAR8 cells. (A) Cell viability following 24-hour incubation with OVCAR8 cells, was assessed at solvent concentrations equivalent to those present in 0.025 mg/mL, 0.05 mg/mL, and 0.01 mg/mL PLGA formulations. (B) Viability assessment of PVA at varying concentrations after 24-hour incubation with OVCAR8 cells. Viability was assessed using the alamarBlue assay. Results were normalized to media controls (n=4).

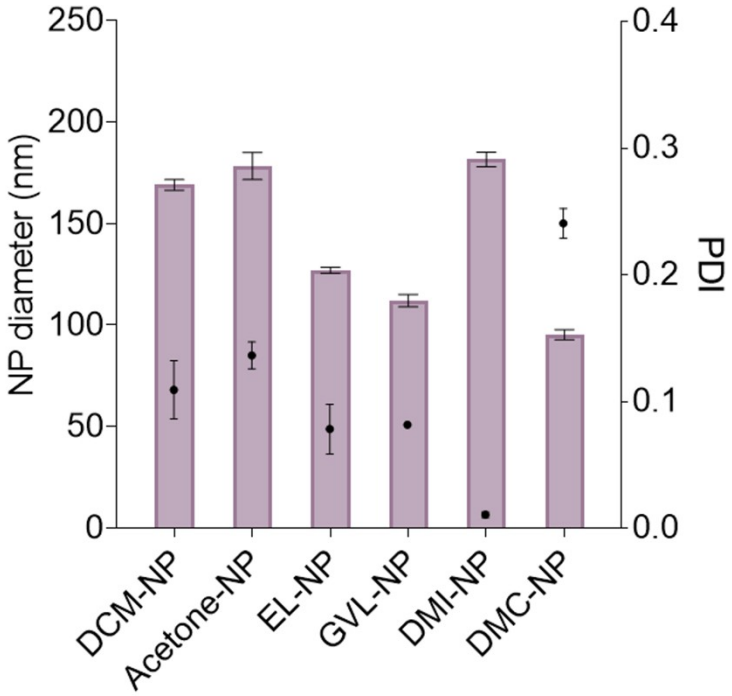

Figure S4. NP diameter and PDI characterization of Cy5-conjugated PLGA-NPs synthesized using green solvents. DLS characterization was performed to obtain NP diameter and PDI. Data are presented as mean ± standard deviation of three technical replicates.

References

1 . N. Boehnke, J. P. Straehla, H. C. Safford, M. Kocak, M. G. Rees, M. Ronan, D. Rosenberg, C. H. Adelmann, R. R. Chivukula, N. Nabar, A. G. Berger, N. G. Lamson, J. H. Cheah, H. Li, J. A. Roth, A. N. Koehler and P. T. Hammond, *Science*, 2022, **377**, eabm5551.
2 . B. Lin, X. Xu, X. Zhang, Y. Yu and X. Wang, *J Biomed Nanotechnol*, 2021, **17**, 1939–1950.