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Spray stability of self-assembled filaments for delivery

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ABSTRACT

Filamentous viruses are common in nature and efficiently deliver – sometimes via aerosol – genetic material, viral proteins, and other factors to animals and plants. Aerosolization can be a severe physicochemical test of the stability of any filamentous assembly whether it is made from natural polymers such as viral proteins or synthetic polymers. Here, worm-like "filomicelles" that self-assemble in water from amphiphilic block copolymers were investigated as aerosolized delivery vehicles. After spraying and drying, fluorophore-loaded filomicelles that were originally ~10–20 μm long could be imaged as 2–5 μm long fragments that survived rehydration on natural and artificial surfaces (i.e. plant leaves and glass). As a functional test of delivery, the hydrophobic pesticide bifenthrin was loaded into filomicelles (up to 25% w/w) and sprayed onto plants infested with two agricultural pests, beet army worm or two-spotted spider mites; pesticidal efficacy exceeded that of commercial formulations. Persistent delivery by the filomicelle formulation was especially notable and broadly consistent with previous intravenous delivery of other drugs and dyes with the highly elongated filomicelles.

1. Introduction

Viruses are nature's self-assembled nanoparticles and exist in various shapes including micron-long filamentous forms. Such viruses include ebola that infects humans and tobacco mosaic virus that infects plants. A current fear is that ebola, with ~50% mortality, could evolve to become more transmissible via vomit or cough-generated aerosols of small droplets (~1–5 μm) or large droplets (5–100 μm) – based on findings that the virus can remain infectious in aerosols for almost 2 h (at ~50% relative humidity and 22 ± 3 °C) [1]. In general, whether self-assemblies bend and break within confining droplets or deactivate (e.g. denature) at the extensive air-water interface of a droplet is unclear, and this poor understanding of the physicochemical stability of filamentous assemblies broadly motivates studies of structure and function using highly controlled chemistries.

Amphiphilic block copolymers are roughly similar in size to proteins that assemble into virus coats but are otherwise chemically more analogous to much smaller and simpler surfactants or lipids (≤ 1000 g/mol) that can also self-assemble in water into a variety of shapes including filaments [2,3]. With the proper ratio of block sizes, worm micelles many microns in length can indeed be made and are sometimes referred to as "filomicelles" when the intent – as here – is to mimic filamentous viruses. On the scale of supramolecular assembly, filomicelles are poised between the molecular level assembly of spherical micelles and so-called giant vesicles of lamellar structure, all of which result – in equilibrium with water – from suitable block ratios of hydrophilic to hydrophobic [3,4]. Non-equilibrium stresses can, however, disrupt micron-size morphologies, and stresses of relevance to various applications generally range from fluid shear to dehydration.

The large molecular weights of block copolymers can impart high thermodynamic stability [5–6] while still permitting degradability and compatibility for drug delivery [7–8]. Poly(ethylene oxide)-*block*-poly (ε-caprolactone) (designated OCL) is an example of interest for biomedical applications: its hydrophobic polyester block is biodegradable and approved by FDA for human application, while the hydrophilic brush of poly(ethylene oxide) (PEO, or PEG) is biocompatible and already in clinical use in parenteral formulations. OCL filomicelles can be sufficiently stable to be injected intravenously and subsequently withstand the fluid shears of blood circulation for days, ultimately enhancing tumor delivery of the hydrophobic drug paclitaxel when compared to spherical micelles [9–10].

Spraying of filomicelles is described here and can also be a major challenge to the stability of these large, soft assemblies. While biomedical applications of sprays are many, we sought to illustrate some broader possibilities through delivery of hydrophobic pesticides

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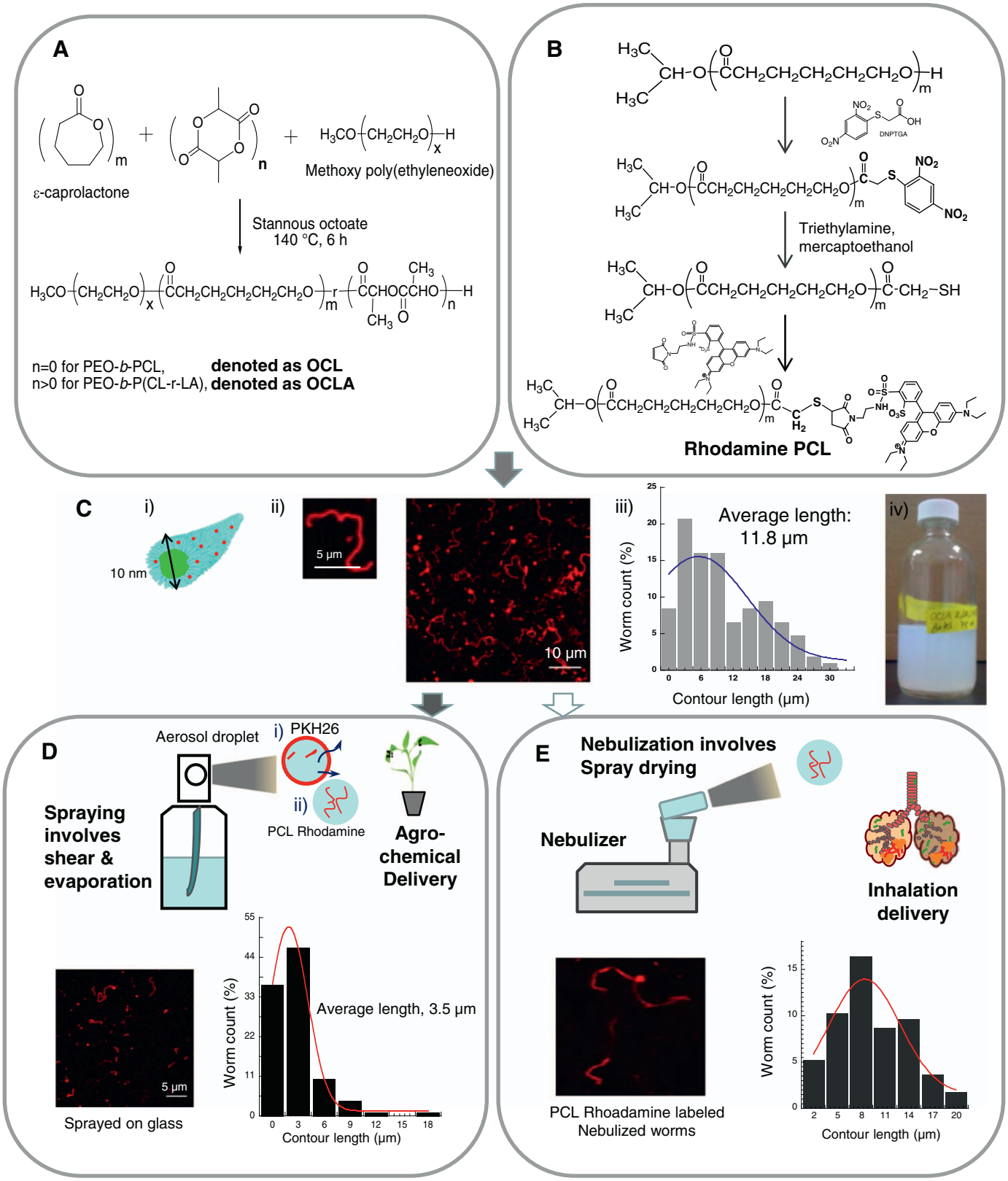

Fig. 1. Spray survival of worm like filo-micelles- A) Synthesis scheme of OCL or OCLA polymer. B) Preparation of Fluorescent dye conjugated PCL (Rhodamine PCL), C) i) Cartoon of a single worm micelle where red spots indicate the integration of Rhodamine PCL. ii) Fluorescent microscopy image of Rhodamine PCL worm micelles confined within a microscope glass slide and cover slip. iii) Contour length distribution of OCLA worm micelles which was measured from several hundreds of worms. Contour length of the worm micelles was measured by straightening and attaching the worms to the positively charged glass slide with the addition of 0.5 mM of NaCl and imaged under fluorescent microscope. iv) The 50 mL bottle is half-filled with an aqueous solution of worm micelles that scatter light, which gives an opaque appearance. D) Spray application of fluorescent dye loaded worm like micelles for the delivery of agrochemicals to the plant: i) Spray droplet redistribution loss of low MW dye PKH 26. ii) Spray droplet with high MW dye PCL-Rhodamine where dye is covalently bound to the worm micelle. Rhodamine PCL labeled OCLA (2, 12) worm micelles sprayed on glass slide with contour length distribution after spray test. E) Nebulization of fluorescent dye loaded worm micelles for the delivery of drugs to the lungs via inhalation: Rhodamine PCL labeled OCLA (2, 12) worm micelles nebulized on glass slide with contour length distribution after nebulization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

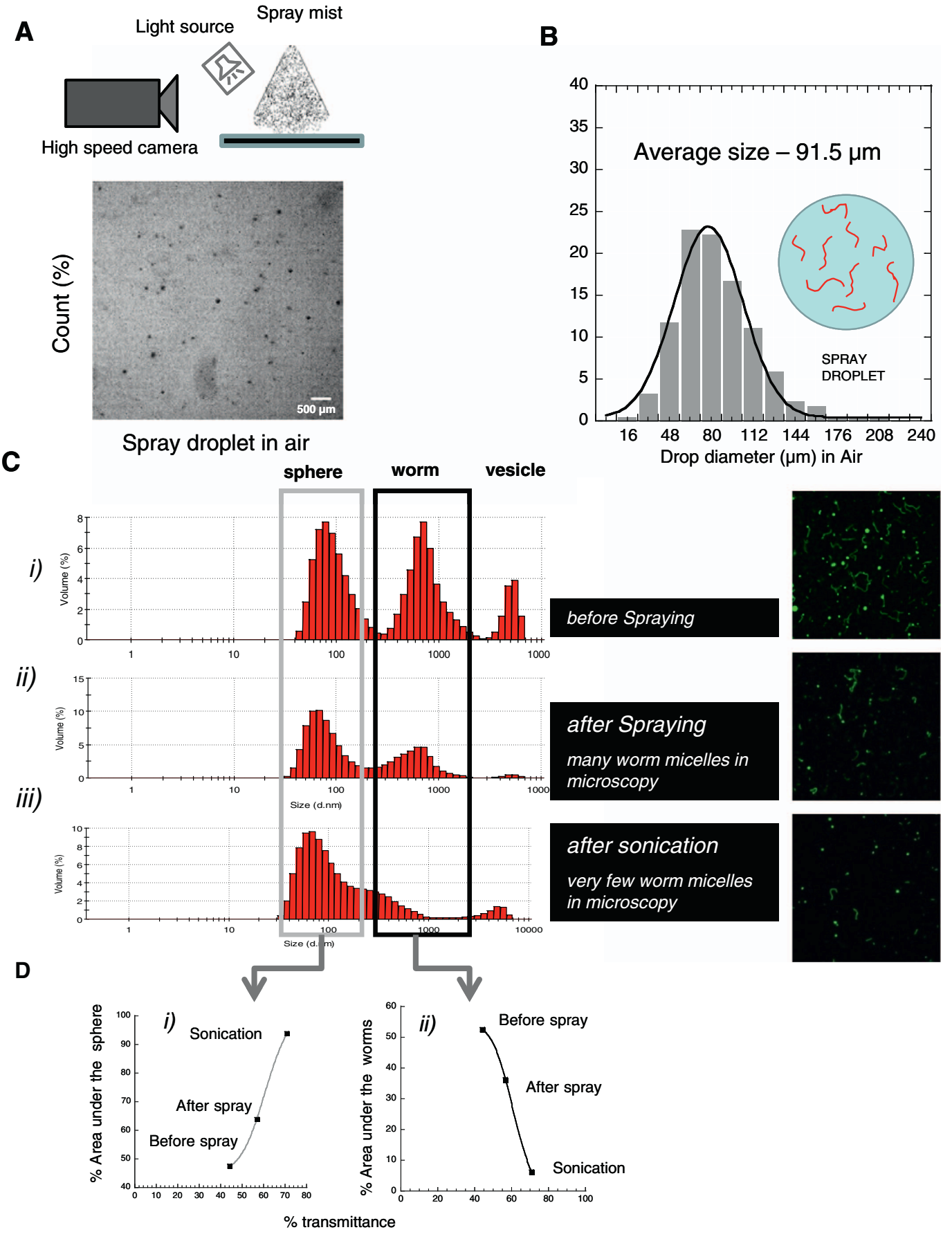

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Fig. 2. Spray droplet size measurement in air and disruptive affect on morphology in solution. A) Imaging set up of spray droplet size measurement by high speed camera (Phantom v7.3) with a capacity of 800 × 600 pixel resolution at 6688 frames-per-second. Image of spray droplets of OCL (2, 12) worm micelle. B) Size distribution of sprayed droplet size after spraying using a house hold sprayer. Data fit by Gaussian for several hundreds of droplets after spraying. C) Assessment of OCLA (2, 12) worm micelle transition to spherical micelle by dynamic light scattering measurement (DLS) at different shear conditions. Co-existence of all three different morphologies i.e., spherical micelles, worm micelles and vesicles right after preparation (i); Worm micelles tend to dissociate and generate spherical micelles after applying shear such as aerosolization (ii) or ultrasound energy (iii). The FM images of the OCLA (2, 12) worm micelles on the far right side of each DLS measurement demonstrate the evidence of worm break down and transformation to spherical micelles after shear applications. D) Dynamic and static light scattering plot of OCLA (2–12) self- assembly demonstrating gradual transition of worm micelles to spherical micelles after various shear conditions. Static light scattering of worm and spherical micelles was assessed by a UV-visible spectrophotometer at 600 nm.

to plants. As commonly applied, pesticides are lost in soil and leach into ground water, with the risk that repeated application can enter the food chain and adversely affect human health [11]. Resistance to agrochemicals has also been observed with hundreds of mite and insect pests [12–14]. More effective and safe delivery of pesticides and other agrochemicals over large areas seemed feasible with filomicelles, provided they can be loaded and the drug retained in the harsh process of spraying.

To solubilize a highly hydrophobic agrochemical such as bifenthrin (BFN), we have used filomicelles made from OCL, copolymerized or not with lactic acid to give OCLA (Fig. 1A), and from poly(ethylene oxide)-*block*-poly(butadiene) (OB). Bifenthrin is a pyrethroid pesticide that has been widely used to protect cotton crops since the early 1980s. It is currently formulated and applied as oil-in-water emulsion droplets, but these can roll off of leaves or are easily washed away by rainfall, irrigation, or wind, compromising drug delivery. Here we show that filomicelles with a fluorescent dye (Fig. 1B) can be made in abundant quantity (Fig. 1C) to be sprayed onto surfaces (Fig. 1D). Importantly, BFN loaded filomicelle formulations of OB and OCL demonstrated significantly higher efficacy against beet army worm (BAW) and two spotted spider mites (TSM) on pinto bean leaves when compared to commercial formulations. The results illustrate the potential for biomedical applications such as inhalation delivery (Fig. 1E).

2. Experimental

2.1. Materials

ε-caprolactone was purchased from Alfa Aesar (Ward Hill, USA), methoxy poly(ethylene oxide) (2000 g/mol), fluorescent PKH26 dye were purchased from Sigma (St. Louis, MO). Stannous octoate was purchased from MP Biomedicals Inc., Germany. Diblock copolymer poly (ethylene oxide)-*block*-poly(1, 2 butadiene) (namely PEO_{91}-PBD_{111}, denoted OB) was purchased (Dorval, Canada). Bifenthrin, Formulations; Brigade 2EC and Talstar Pro; Broccoli and Pinto bean plants (4 weeks of age) and PVC column filled with Princeton sandy loam soil were kind gift from FMC Corporation (Ewing, USA). All organic solvents were analytical grade from Fisher Scientific. Fluorescent dye, Rhodamine Red® C_2maleimide and near IR dye, DiR were purchased from Invitrogen Inc. (USA).

2.2. Synthesis of OCL and OCLA diblock copolymers

Amphiphilic di-block copolymers, PEO_{45}-*b*-PCL_{105} (OCL 2,12) and PEO_{45}-*b*-P(CL_{92}-*r*-LA_{21}) OCLA (2,12) were synthesized by standard ring opening polymerization of ε-caprolactone and *DL*-lactide using Me-PEO (2000 g/mol) as initiator and stannous octoate as catalyst (Fig. 1A). Briefly, the synthesis of OCL (2, 12), freshly distilled ε-caprolactone (2.5 g, 0.0219 mol), Me-PEO (2000 g/mol) (1 g, calculated based on the desired PCL MW) and stannous octoate (15 mg, 3.7×10^{-5} mol) were placed in previously flamed ampoule (10 mL), nitrogen purged and sealed under vacuum. The polymerization reaction was allowed to proceed for 4 h at 140 °C in oven. The reaction was terminated after cooling the ampoule to room temperature. For the synthesis of OCLA (2, 12) copolymer, a mixture ε-caprolactone and DL-lactide at 90:10 ratio (M/M) were polymerized together initiated with Me-PEO (2000 g/mol) (0.4166 g).

2.3. Estimation of block ratios by ^1H NMR spectroscopy

The synthesis of polymers was verified from ^{1}H NMR spectra collected using a Bruker NMR360 spectrometer. The block ratios were calculated from the integrals at 3.6 ppm (methylene protons of PEO) and 4.05 ppm (methylene protons of caprolactone). The percentage of each monomer incorporated into polymer chains was analyzed by ^{1}H NMR spectroscopy in $CDCl_3$, where the methylene protons ($-COOCH_2-$) of PCL appear at ~4.05 ppm, and the methine proton signal ($-COO-CHCH_3-$) of polylactide appears at ~5.15 ppm.

2.4. GPC measurement

The molecular weight distributions of all the synthesized polymers were determined by gel permeation chromatography using a Waters system equipped with a Waters 1215 binary pump and Waters 2414 refractive-index detector. Separation was performed using Styragel HR2 column, calibrated with polystyrene standards and tetrahydrofuran as solvent.

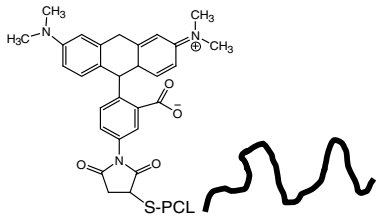
2.5. Synthesis of rhodamine conjugated PCL (PCL-Rho)

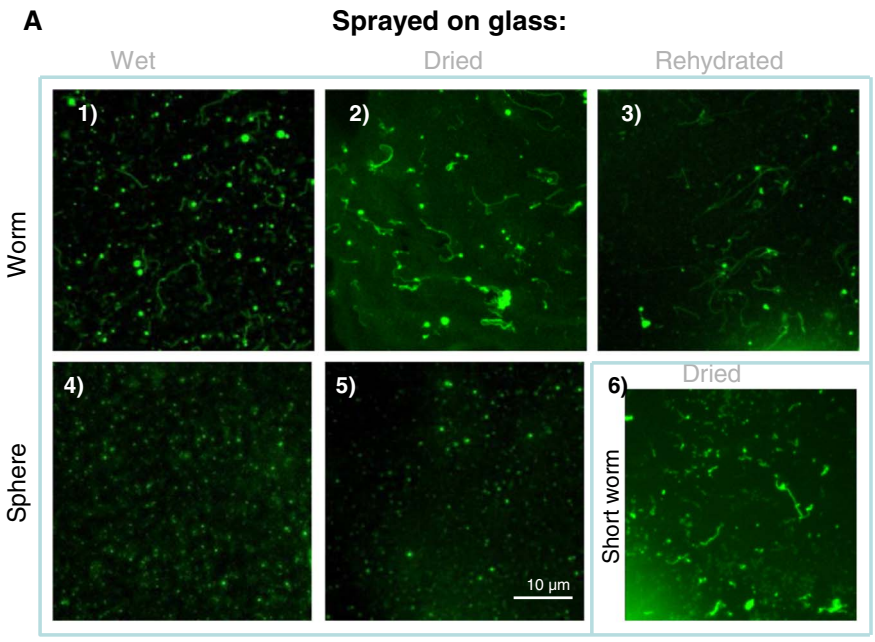
The synthesis was performed according to the method described previously [15] (Fig. 1B). Briefly, thiol terminated PCL (PCL-SH) (7700 g/mol, 36 mg, 4.7×10^{-3} mmol.) was taken in a clean 5 mL RB flask and dissolved in 1 mL of freshly distilled DCM. A solution of 1.2 equivalents of rhodamine maleimide (680.8 g/mol, 3.82 mg, 5.61×10^{-3} mM) in anhydrous DMSO (50 μL) was added to the RB flask under argon condition. The reaction mixture was stirred and continued overnight under argon. The dye conjugated polymer (PCL-Rho) was separated from unreacted rhodamine maleimide by dialyzing (Spectrapor membrane, MWCO: 3500) the reaction mixture against DMSO for 12 h followed by dialysis against DI water for additional 48 h. The purified polymer, PCL-Rho in dry form was obtained after freeze-drying.

2.6. Preparation of filo and spherical micelles

Filomicelles from OCL (2, 12) and OB (4, 6) polymers were prepared in large batches (250 mL) by solvent evaporation method as described previously [4]. Briefly, OCL and OB polymers (0.025 mM) were dissolved in 5 mL of chloroform with gentle shaking and prepared polymer solutions were added to 250 mL of DI water in a clean 1 L flat bottom glass bottle. Micellization was induced by slow evaporation of chloroform by stirring the mixture at room temperature under gentle speed (~110 rpm). The stirring was continued for 60 h for complete removal of chloroform. The final polymer concentration in filomicelle solution was 100 μM. Spherical micelle from OCL (5, 6) was also prepared using identical method at final polymer concentration of 100 μM in DI water.

Filomicelles (10 mL) labeled with PCL-Rho for spraying were prepared by dissolving PCL-Rho (8380 g/mol, 35.5 nM) along with OCL (2 − 12) (14,000 g/mol, 710 nM) in chloroform (500 μL) and subsequent addition to 10 mL of DI water in an amber glass vial. The mixture was stirred at gentle speed (~110 rpm) for 48 h for complete removal of chloroform. The glass vial was covered with aluminum foil to prevent photo bleaching of the fluorophore. Spherical micelles





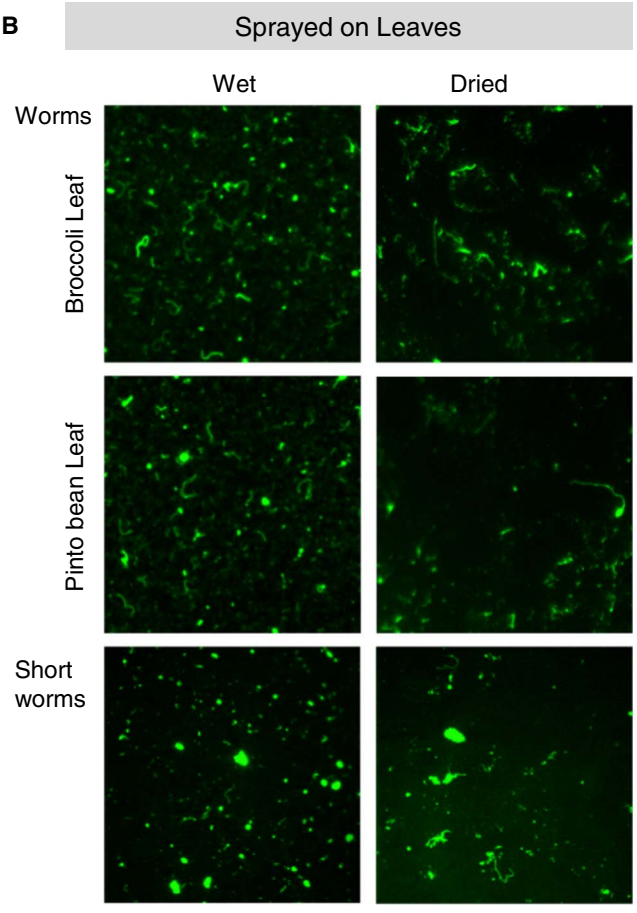


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Fig. 3. A. Survival of sprayed OCL worm micelles after drying and rehydration. 1st row: OCL (2,12) worm micelles loaded with PCL conjugated hydrophobic fluorescent dye (Rhodamine-PCL) sprayed on glass slide; air dried for 2 h and rehydrated with DI water. 2nd row: OCL (5, 6) spherical micelles sprayed on glass slide and air dried (Images 4 and 5). Image 6 shows OCL (2, 12) short worm micelles (probe sonicated) and air dried. Scale bar indicates 10 μm. B. Worm micelles sprayed on leaf surface and survival after drying: Rhodamine PCL loaded OCL (2, 12) worm micelles and sonicated short worm micelles (bottom row) sprayed on two different kinds of leaves; Pinto bean and Broccoli leaf and air dried on leaf surface. Scale bar indicates 20 μm.

(10 mL) from OCL3 were prepared by following identical procedure.

Short worm (SW) micelles were obtained by sonicating PCL-Rho labeled OCL filomicelles using Fisher 60 Sonic Dismembrator equipped with Fisher Ultrasonic Converter (Fisher Scientific) for 25 pulses at 1 s/pulse.

2.7. Fluorescence microscopy imaging of filo and spherical micelles

50 μL of polymeric micelle solution (100 μM) was taken into an eppendorf tube and labeled with 0.2 μL of 0.2 mM hydrophobic fluorescent dye (PKH26, sigma). An aliquot of 2 μL of dye labeled micelle sample was placed on a microscope slide and covered with round cover-slip (18 mm × 1 mm), pressed gently and sealed with vacuum grease on the edges. The micelle sample was imaged using a 60× lens with oil on Olympus IX71 microscope equipped with a Cascade 512B camera

In order to image the sprayed micelles, sprayed samples on bare glass slide or on leaves on glass slide was covered with a rectangular (22 × 22 mm) cover-slip pressed gently and the edges were sealed with vacuum grease. The samples were imaged using a 60× lens with oil at on Olympus IX71 microscope equipped with a Cascade 512B camera.

2.8. Contour length distribution of filomicelles

An aliquot of 50 μL of fluorescently labeled filomicelle solution (100 μM) was diluted to 400 μL with DI water in an eppendorf tube. 10 μL of NaCl solution (100 mM) was added to the filomicelle solution and mixed gently. Salt mixed 3 μL of diluted filomicelle solution was dropped on a microscope cover-slip (diameter: 18 mm), pressed hard to make a thin film, sealed the slide with vacuum grease and imaged by an Olympus IX71 microscope using 60× objective equipped with a CCD camera. The addition of salt immobilizes and sticks the individual filomicelle to the glass surface facilitating the measurement of filomicelle contour length. For each sample, several hundred filomicelles from 10/12 frames were measured using ImageJ program.

2.9. Spray droplet size measurement with high speed camera

The experimental apparatus used for filomicelle spray droplet size measurements consisted of a high speed camera (Phantom v7.3, NJ, USA), two high voltage light source (750 W each) and a PC equipped with phantom PCC software to control the camera and process images (Fig. 2A). The camera images up to 6688 frames-per-second (3 GPx/s) with an adjustable shutter speed down to 1 μsec (1/1,000,000 s). The camera provides full frame 4:3 aspect ratio, 14-bit image depth (standard) with CMOS sensor composed of 800 × 600 pixels resolution at 22 μm pixel size. The video was taken during spraying of plain PCL filomicelle and the captured image was processed with ImageJ software to calculate the droplet size.

2.10. Spray survival of filomicelles

Spray survival of OCL filomicelle was conducted using a CO_2 agricultural backpack sprayer (FMC Corporation). A typical insecticide or fungicide (hollow cone) nozzle was used with a 100 mesh screen at set pressure of 40 psi. The nozzle was held at about 18–20″ from the receiving surface during spraying and spray application was performed into petri dish. The spray sample was collected for analysis under FM after labeling with PKH 26 dye. In order to assess the spray survival on plant leaves, a single leaf (e.g., Broccoli and Pinto bean) was cut in about 4 cm^2 and stuck on a glass slide surface with silicone vacuum grease. PKH 26 and PCL-Rho labeled different micelle formulations: worm, sonicated short filomicelle and spheres were sprayed on bare glass slides as well as glass slides covered with leaves.

2.11. Drying Rewetting analysis of filomicelles

Red fluorescent dye (PKH26, Sigma) and PCL-Rho (5 mL) labeled OCL (2, 12) and OB (2, 12) filomicelle solution (100 μM) was taken in a 100 mL spray bottle (Nalgene Labware, USA) and sprayed on microscope bare microscope glass slide as well as on leaf surface on glass slides. PCL-Rho labeled filomicelles were air dried by leaving the slides in dark for about 2 h. The dried, sprayed, fluorescently labeled micelle samples were observed for survival after drying by imaging under fluorescent microscopy. Rehydration test was performed by spraying plain DI water on dried fluorescently labeled filomicelle samples and imaging under fluorescent microscopy.

2.12. BFN encapsulation in OCL and OB filomicelles

Varied amount of BFN (1, 2.5, 5.0, 10 and 20 mg) was solubilized in 3 mL of ethanol and added to the freshly prepared filomicelle solutions (10 mL) in a glass vial, stirred for 30 min at RT and left overnight with closed cap. Next day, BFN loaded filomicelle formulations were dialyzed (Spectrapor membrane, MWCO: 3500 KD) against DI water for 6 h to remove ethanol and small fraction of unloaded BFN. After dialysis, the samples were centrifuged at 3000 rpm to precipitate unloaded BFN aggregates and some larger polymer aggregates. The preparation of BFN loaded OCL filomicelle formulation is illustrated in Supplementary Fig. S1.

2.13. HPLC analysis to quantify BFN loading level in filomicelle formulation

A Shimadzu HPLC system (Shimadzu Corporation, Japan) equipped with an inline DGU-20A3 vacuum degasser, LC-20AB binary high pressure pump, SIL-20 AC high speed auto-sampler and a pinnacle® DB C18 reverse-phase column (4.6 × 150 mm) was used to detect and quantify BFN in filomicelle formulation. Briefly, 20 μL of sample was injected to the HPLC system and eluted by a mobile phase consisting of acetonitrile and water with 0.1% of trifluroacetic acid at a flow rate of 1 mL/min at 35 °C. In gradient elution, the starting acetonitrile concentration was 70% and increased to 80% within 15 min at a constant rate. The detection was performed at λ = 220 nm and quantified using a diode array detector (SPD M20A, Shimadzu). A standard curve was prepared from BFN at concentration of 0.001, 0.01, and 0.05 mg/ml. Data was acquired and processed with LC solution chromatography software from Shimadzu Corporation. BFN loaded OCL and OB filomicelles were mixed with acetonitrile and water at a ratio of 70:30 to break the micelle structure and solubilized the BFN followed by the HPLC analysis using the standard curve described above. BFN loading level and encapsulation efficiency were calculated based on the following expressions:

$$BFN\ loading\ [weight/weight\ ](\%) = \frac{amount\ of\ loaded\ BFN\ in\ mg}{amount\ of\ copolymer\ in\ mg} \times 100$$

$$Encapsulation\ efficiency\ (\%) = \frac{amount\ of\ loaded\ BFN\ in\ mg}{amount\ of\ BFN\ added\ in\ mg} \times 100$$

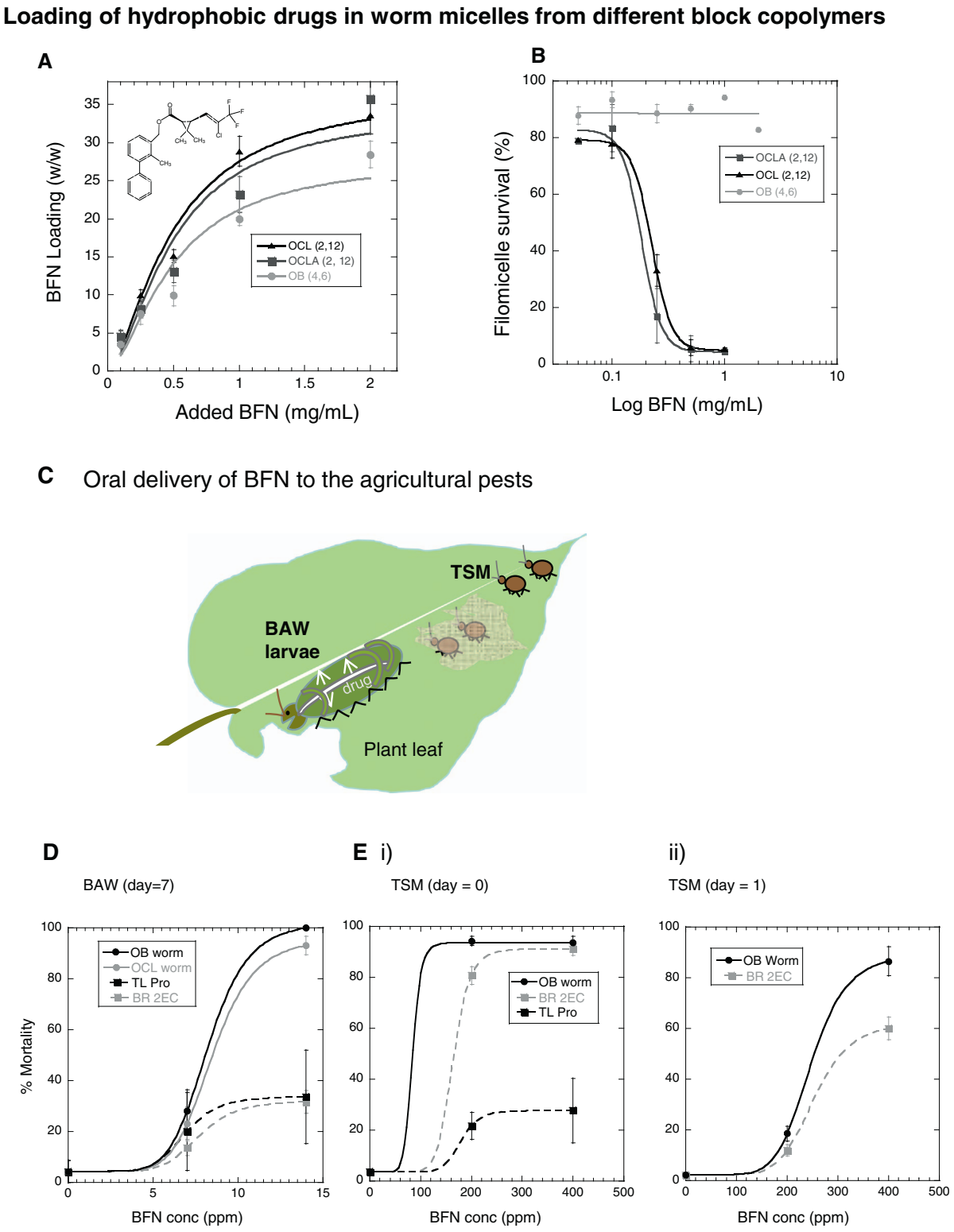

Fig. 4. A) Encapsulation of a pesticide, BFN in polymeric filomicelles. Bifenthrin (MW 422.874 g mol^{-1}) is a 4th generation pyrethroid insecticide. Apparent loading (w/w) of Bifenthrin in OCL 2–12 and OCLA 2–12 worm micelles as a function of Bifenthrin added (mg/mL). B) Plot showing the survivability of worms after loading BFN. OCL and OCLA, both worms didn't show adequate survival after BFN loading at higher concentration but addition of Bifenthrin at 0.1 mg/mL was found not affecting the worm structure. C) Cartoon for oral delivery of a pesticide to agricultural pests after BFN loaded OCLA (2, 12) worm micelle formulation is sprayed on the plant leaves. The BAW larvae chew the whole leaf and BFN is released in the digestive system to be effective. A lower concentration of drug is required to control BAW because TSM mites feed only on leaf surface and ingest only the residual amount of drug left on the leaf surface. D) Residual efficacy of OCL (2,12) and OB (4, 6) worm micelle formulations compared to commercial formulations: Talstar Pro and Brigade 2EC at two different BFN concentration (7 ppm and 14 ppm) against BAW after 7 days of pesticide application. The insects received 72 h of exposure time with leaves after spray application. All data points for BAW study represent the average ± SD (n = 4 groups) comprising 18–20 insects per group. E) Efficacy against TSM at two different concentrations of BFN (200 ppm and 400 ppm) after 0 day (i) and 1 day (ii) of pesticide application. The insects received 96 h and 72 h of exposure time for 0 day and 1 day experiment respectively. All data points for TSM study show the average ± SD (n = 4 groups) comprising of 40–80 insect in each group. a indicates significant difference from b (P < 0.01, one way ANOVA).

Table 1
Solubilization of Bifenthrin by worm micelles.

Polymer[a]	Morphology	Solubilization (μg/mL) ± SD	Effective loading[b] % (w/w) ± SD	Encapsulation efficiency (%)[c]
OCLA (2,12)	Worm	77 ± 24	4.2 ± 1.2	77 ± 10.4
OCL (2,12)	Worm	88 ± 15	4.7 ± 1.5	88 ± 6.0
OB (4, 6)	Worm	568 ± 73	28.5 ± 3.0	29 ± 3.7

[a] The full name of the polymers: OCLA; poly (ethyelene oxide)-*block*-poly(ε-caprolactone-*random*-D,L Lactide), OCL; poly (ethyelene oxide)-*block*-poly(ε-caprolactone), OB; poly (ethyelene oxide)-*block*-poly(1,2 butadiene); The numbers inside the parentheses indicates the molecular weight of each polymer block in KD.
[b] Effective loading is the amount of bifenthrin loaded into OCL based worm micelles without breaking the worm structure.
[c] Encapsulation efficiency is calculated: solubilized Bifenthrin/mass of initially added Bifenthrin.

2.14. Determine efficacy against beet army worm under simulated commercial conditions

The efficacy and residual efficacy of filomicelles against two agricultural pests (two spotted spider mites and beet army worm) was performed under simulated commercial conditions in FMC agrochemical research facility (Ewing, NJ, USA) (Supplementary Fig. S2). Briefly, host plant pinto bean was selected so that at least five 1″ leaf disks from the primary leaves of each plant (~12 days after planting) can be obtained. Spray application on pinto bean plant was performed using 20 mL of test formulation to treat 28 plants at a time using a DeVries traveling boom sprayer at a spray volume of 280.5 L/ha (40 psi) equipped with a hollow cone spray tip. BFN loaded two filomicelle formulations (OCL & OB) along with two commercial formulations (Tal Pro & BR 2EC) were tested at BFN concentrations of 200 & 400 ppm for TSM and 7 & 14 ppm for BAW. After spray application, the plants were allowed to air dry and transferred to the green house until ready for infestation with pests. The plants were watered by surface (ebb & flow) irrigation. To assess the efficacy against BAW, the leaves were infested with larvae after 3, 7 and 10 days of spray application. BFN formulation treated leaf samples (1 in. leaf disks) from each plant were collected and placed (adaxial surface up) on moisten filter paper in petri dish. BAW larvae (two/petri dish) of 2nd in star were carefully placed with fine tipped artist's brush and ten larvae were used in each treatment group (n = 3 groups). The dishes were held in a growth chamber for 72 h, 40–60% of relative humidity and photoperiod of 12:12 (light: Dark). The numbers of dead, moribund larvae present on the leaf disks for each group were recorded. To assess the efficacy against TSM, adult mites (n = 50/75, by visual estimation) were placed on the adaxial surface of a leaf on treated pinto bean plant. After 72 h of exposure on leaves, the number of live adult mites present on the infested leaves was recorded for each group for evaluation of the formulation efficacy.

3. Results and discussion

3.1. Synthesis of OCL and OCLA polymers and preparation of filomicelles

Ring opening polymerization (ROP) was used to synthesize OCL (2, 12), where 2 and 12 are the sizes of PEO (2000 g/mol) and PCL (12,000 g/mol) block (Fig. 1A). GPC analysis of the synthesized polymers revealed narrow polydispersity indices for both OCL and OCLA polymers (Supplemental Table S1, Supplemental Fig. S3). To image filomicelles, a small fraction of rhodamine-PCL (Fig. 1B) was sometimes added to formulations with rhodamine-PCL made by Michael addition reaction between maleimide-rhodamine and thiol (SH) functionalized PCL (MW 7500 g/mol). Since lactides and lactones both undergo similar ROP processes via a coordination insertion [16–17], we utilized the same technique for random copolymerization of CL and DL-lactide (LA) by incorporating 10 mol% of LA to synthesize poly(ethylene oxide)-*block*-poly(ε caprolactone-*r*-D,L Lactide) (Fig. 1A). This copolymer is denoted as OCLA (2, 12), where 2 and 12 are the sizes of PEO (2000 g/mol) and poly(CL-LA) (12,000 g/mol). NMR showed the percent of ε-caprolactone and lactide units in the polymer chains (Supplemental Table S1) corresponded well with the monomer feed ratios and the percent conversion of both monomers was ~98% to give respective degrees of polymerizations of 91 and 16 for CL and LA in poly(CL-LA).

Filomicelles assembled with amphiphilic degradable polymers OCL (2, 12), OCLA(2, 12) and inert polymer OB(4, 6) were prepared by solvent evaporation as described previously [4], but with the preparation method scaled up from 1 mL to 300 mL for spray application. All three polymers made giant and stable filomicelles with a well resolved contour length (> 2 μm) observed in fluorescence imaging (Fig. 1C and Supplemental Fig. S4). Contour length distributions were fit (Fig. 1C–E) to a Zimm-Shulz model, $f = b[L.\exp.(-2L/L_n)]$ where b is a pre-factor and L_n is the number average contour length [18]; for both OCL(2, 12) and OCLA(2, 12), the average length was ~12 ± 3 μm (Supplemental Fig. S4). With time, degradable giant filomicelles are expected to shorten toward spherical micelles [10]: after 3 months storage at room temperature, the average lengths for OCLA and OCL filomicelles were both ~7 μm (Supplemental Fig. S4). Stability is relevant to storage and to survival in drug delivery applications [19].

3.2. Sprayability of filomicelles

Suspensions of filomicelles of OCL and OCLA polymers were sprayed by a CO_2 agricultural back pack sprayer through a cone, and high speed imaging [20–22] was used to quantify average droplet sizes of 91 μm (Fig. 2A,B). In pesticide delivery, measurement on spray droplet size is critical since droplet size plays a significant role on the distribution and coverage of pesticide on foliage thereby reducing environmental pollution, repeated application to enhance pesticide efficacy [23–24]. Spraying into solution was followed by imaging and dynamic light scattering (DLS). Filomicelles were shortened to 3–8 μm and were reduced in number based on both imaging and DLS (Fig. 2C,D). Similar results were obtained with a standard laboratory sprayer (Nalgene, USA), and sonication was used to confirm total disruption could be easily achieved [19]. The filomicelles partially withstood the shear of spraying through a nozzle and also retained the integrated fluorophore.

Long term efficacy of a pesticide requires formulation stability on a leaf surface for the pest to encounter it. Spraying of filomicelles onto glass slides and plant leaves was studied in both wet and dry states (Fig. 3A,B). Filomicelles of OCL(2, 12) dyed with hydrophobic PCL-Rho remained fluorescently visualizable even after drying and rehydrating. The hydrophobic fluorescent dye, PKH 26 is much smaller (961 g/mol) and although visible in aqueous environment, the morphologies were not detectable after drying. This is consistent with loss of dye to cell membranes or lipid sinks in past studies [25]. Nonetheless, polymeric micelles in dried states on surfaces are well documented by microscopies such as TEM and AFM [26–27]. With PCL-Rho labeled spheres, fluorescent dots were evident but not any elongated structures, whereas sonicated short filomicelles (OCL-SW) showed some filamentous morphologies (Fig. 3A). Robustness of filomicelle against 'rain' after drying was further assessed by rehydrating the dried worms on glass slides, and once again elongated filomicelles were evident (Fig. 3A).

Survival of filomicelles on plant leaves was assessed next. Whether they adhere or not is important because loss of applied pesticides from

plant leaves leads to their repeated application and more off-target effects [28–29]. OCL filomicelles, short worms and spheres loaded with PCL-Rho were sprayed onto Broccoli leaves and Pinto bean leaves and later assessed for their survival after air drying for several hours. Filomicelles were again found to survive (Fig. 3B). Survival of filomicelles on the leaf surfaces therefore seems promising for delivery of bioavailable pesticides.

3.3. BFN loaded filomicelles

Solubilization of hydrophobic drugs such as Cucurbitacin I [30], Cyclosporine A [31], as well as TAX [32] is generally enhanced by polymeric micelles, and these paclitaxel-loaded filomicelles have been used to shrink tumors [9,33–35],. The pesticide BFN is a pyrethroid insecticide that also has low water solubility (< 0.01 mg/mL). BFN was readily loaded into filomicelles (Fig. 4A) from organic solvent phase during dialysis (Supplemental Fig. S1). Loading levels achieved with OCL and OCLA filomicelles were 4–5% w/w (drug/polymer) (Table 1), with microscopy showing that efforts to load higher BFN (at > 0.1 mg/mL) would disrupt the elongated filomicelle morphology (Fig. 4B). Such disruption was not observed previously with other hydrophobic drugs such as paclitaxel and does not occur with filomicelles of OB that have pure hydrocarbon in their cores rather than any polarizing, oxygen moieties. The fluoro-carbon group of BFN will tend to generate a separate phase that is immiscible with hydrocarbons as well as water [36], and the two oxygen groups in BFN structure could also confer disruptive surfactant behavior [37]. Efficacy studies of BFN-loaded filomicelles thus required using the 4–5% w/w drug formulations.

3.4. Pesticidal efficacy of BFN loaded filomicelle formulation

Once sprayed onto leaves, the BFN loaded filomicelles were assessed against two different agricultural pests (Fig. 4C and Supplementary Fig. S2). The first studied was the beet army worm (BAW), which is a major agricultural pest affecting cotton crops plus a variety of vegetables and which has developed resistance to many chemical pesticides. The larvae feed primarily on foliage, causing severe damage to plants. Pinto bean plants were treated with filomicelle formulations of BFN in OCL or OB filomicelles and compared to DI water as well as commercial formulations of BFN: BR2EC and TL-Pro, which are micro-emulsion formulations. Pinto bean leaves were infested with BAW larvae (2nd instar) 7 days after treatment to assess the residual efficacy of the various formulations, with efficacy estimated by counting the dead and morbid larvae 72 h later. Preliminary studies at 3 days after treatment suggested all formulations performed equally well to control the BAW infestation and differences at 10 days were not significant (Supplementary Fig. S5). However, filomicelle formulations at 7 days after treatment were about 3-fold higher pesticidal efficacy at 14 ppm of BFN versus commercial formulations (Fig. 4D: $p < 0.0001$, one way ANOVA, 3 groups with 20 larvae in each group).

The two-spotted spider mite (TSM) is a herbivorous crop destroying pest worldwide and is the most damaging mite for cotton growth [38–39]. These mites feed on the undersides of leaves, which are the major sites of photosynthesis [40–41], but spraying is applied to the tops of leaves, which can limit efficacy. Pesticidal efficacy required high doses of > 200 ppm of BFN, and so only OB filomicelles formulations could be tested. Efficacy was assessed on 0, 1 and 4 days after treating the pinto bean plants with BFN formulations. The percent mortality was assessed from the number of live mites present on the infested leaves 72 h post-exposure. The filomicelle formulation demonstrated slightly better pesticidal efficacy over the commercial formulation BR2EC at 200 ppm, with mortality of 94% for OB versus 81% for BR 2EC ($P < 0.02$, one way ANOVA), whereas both formulations were equally effective at 400 ppm on day 0 of BFN treatment (Fig. 4D-(i)). The TL-Pro formulation was much less effective. At 1 day after spraying, the two effective formulations were less so at 200 ppm, but the 400 ppm tests again showed the filomicelles formulation to be slightly better (Fig. 4D-(ii)) ($P < 0.0001$, one way ANOVA).

Lastly, we tested permeation into soil, which can be relevant to delivery of BFN to pests such as larvae on the soil surface as well as to environmental contamination. Filomicelles labeled with near IR fluorescent dye, DiR, were found to not penetrate more than ~3 cm through soil (Supplementary Fig. S6).

4. Conclusion

Filomicelles self assembled for scale up from amphiphilic block copolymer show remarkable stability after spraying onto surfaces including plants. The hydrophobic pesticide, BFN, is perhaps representative of other hydrophobic pesticides and was shown to be stably solubilized at low loading into OCL, OCLA and OB filomicelles. In biological efficacy assessment, BFN loaded filomicelle formulations demonstrated pesticidal efficacy equal to or greater than that of commercially available formulations, when tested against two common agricultural pests. While this study highlights the potential application of polymeric filomicelles to deliver pesticides in agriculture, it is of course only a feasibility test. More importantly it illustrates the broad drug delivery potential of filomicelles inspired by filamentous viruses that infect plants and animals. Finally, filovirus stability and functional infectivity in aerosols are thus more understandable based on principles revealed by these simpler physicochemical self-assemblies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2017.05.026.

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