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Tunable delayed controlled release profile from layered polymeric microparticles

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Abstract

Composite microparticles (MPs) with layered architecture, engineered from poly(L-lactic acid) (PLLA) and poly(D,L-lactic-co-glycolic acid) (PLGA), are promising devices for achieving the delayed release of proteins. Here, we build on a water-in-oil-in-oil-in-water emulsion method of fabricating layered MPs with an emphasis on modulating the delay period of the protein release profile. Particle hardening parameters (i.e. polymer precipitation rate and total hardening time) following water-in-oil-in-oil-in-water emulsions are known to affect MP structure such as the core/shell material and cargo localization. We demonstrate that layered MPs fabricated with two different solvent evaporation parameters not only alter polymer and protein distribution within the hardened MPs, but also affect their protein release profiles. Secondly, we hypothesize that ethanol (EtOH), a semi-polar solvent miscible in both the solvent (dichloromethane; DCM) and non-solvent aqueous phases, likely alters DCM and water flux from the dispersed oil phase. The results reveal that EtOH affects protein distribution within MPs, and may also influence MP structural properties such as porosity and polymer distribution. To our knowledge, we are the first to demonstrate EtOH as a means for modulating critical release parameters from protein-loaded, layered PLGA/PLLA MPs. Throughout all the groups in the study, we achieved differential delay periods (between 0 – 30 days after an initial burst release) and total protein release periods (~30 – >58 days) as a function of solvent evaporation parameters and EtOH content. The layered MPs proposed in the study potentially have wide-reaching applications in tissue engineering for delayed and sequential protein release.

Graphical abstract

Solvent evaporation parameters and ethanol content during PLGA/PLLA microparticle (MP) fabrication affect protein distribution and MP structure, thereby altering the protein release profiles.

[†]Footnotes relating to the title and/or authors should appear here. Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

1. Introduction

Polymeric biodegradable constructs enable the localized delivery of therapeutics while circumventing deleterious side effects associated with systemic administration, and negating the need for the recovery of a non-degradable device. Poly(L-lactic acid) (PLLA) and poly(D,L-lactic-co-glycolic acid) (PLGA) are among the most prominent materials used for drug carriers as they are both FDA-approved, can encapsulate a wide range of molecules, are biodegradable, and have easily metabolizable degradation products.¹ Moreover, these polymers insulate the encapsulated cargo from environmental factors, thereby decreasing specific and non-specific degradation, a critical parameter for bioactive protein delivery.^{2–4} PLGA/PLLA delivery devices may be engineered into a variety of shapes/sizes such as sponges in the macro-scale, as well as particles and fibers in the micro-/nano-scales for less invasive implantation within target tissues. Single polymer PLGA or PLLA micro-/nanoparticles are among the most common types of carriers studied for sustained release of many types of cargo including small molecule drugs, peptides, proteins¹, anaesthetic agents⁵, viruses⁶, narcotics⁷, and others.⁸ Yet, these traditional single polymer PLGA or PLLA particle systems have inherent limitations that include a high initial burst release and relatively low encapsulation efficiencies.^{9–11}

Layered (i.e. core-shell) composite PLGA/PLLA microparticles (MPs) aim to address the shortcomings of the single polymer MP systems.^{12,13} A number of studies report that layered composite PLGA/PLLA MPs significantly reduce the high burst release while achieving high loading efficiencies (>98%).^{11,14–16} Such composite layered structures may be fabricated via complex electrospraying techniques^{17,18} or through a one-step production consisting of emulsion followed by solvent extraction/evaporation (ideal for hydrophobic cargo).^{12,13} The one-step emulsion technique may also be adapted to encapsulate hydrophilic proteins (termed water-in-oil-in-oil-in-water; W/O₁/O₂/W)^{19,20} to improve the bioactivity of encapsulated cargo during both particle fabrication and through the various phases of the release.^{14,21} In this process, conventional emulsion techniques are used with two immiscible polymers (i.e. PLGA and PLLA), where the protein payload in aqueous media is emulsified in either one or both polymer phases. The polymer constituents phase separate during solvent evaporation/extraction and adopt one of three general configurations:

complete, partial or no engulfment of one polymer over the other.^{12,13,22} Entrapped cargo is expected to preferentially complex with one polymer over the other, based on polymer-cargo and cargo-solvent interactions.¹⁵ In addition to layered MPs, multi-phase particles with hierarchical structures also generate unique release profiles (i.e. delayed release of encapsulated cargo) that are partially dependent on polymer content and cargo localization.^{14–17,23–26} Subsequent cargo release profiles from either layered or multi-phase MPs may be altered by tuning the shell material/thickness and distribution of cargo within the MP.

Delayed release proteins are of great interest as their spatiotemporal presentation is often critical for a desired biological outcome.^{27–29} For example, delayed release of the chemoattractant, stromal cell-derived factor-1 (SDF-1), may amplify and sustain endogenous stem cell recruitment to enhance regeneration of lost tissues after neural injury (e.g. after a stroke or traumatic brain injury).^{30–34} Other strategies include orchestrated co-delivery of proteins that mimic endogenous patterns, which may lead to enhanced, or synergistic therapeutic effects.^{35,36} Sequential delivery of multiple agents is also being studied to target drug-resistant cancer cells.³⁷ Composite PLLA/PLGA MPs with layered architecture have been widely investigated for the delayed release of small molecule drugs. However, the extension of these devices for delayed protein release and robust means of tuning controlled release parameters (i.e. delay period, release rate) is still relatively limited. Altering shell thickness/material of the layered MPs is the most common technique for tuning release profiles from layered PLGA/PLLA MPs.^{17,18,20} Although newer techniques that use bi-/tricapillary electrosprayers impart greater control over particle population (diameter and polydispersity) and shell thickness compared to the W/O₁/O₂/W process, the requirement for specialized equipment impedes their widespread adoption.^{17,18} Thus, in this work, we describe novel methods that build on the straightforward W/O₁/O₂/W emulsion to tune the distribution of hydrophilic protein within composite PLGA/PLLA MPs.^{14,19} We probed two main fabrication parameters to achieve significantly altered protein distribution within composite PLGA/PLLA MPs. First, we hypothesized that solvent flux from the dispersed oil phase and total time of solvent evaporation affects the organization of the PLGA and PLLA phases within the hardened particle, impacting the overall protein release profile. Although a number of studies have elucidated the effects of solvent evaporation rate and time on the structure of layered MPs^{12,13,23,38}, to our knowledge, we are the first to determine the effects of protein release profiles. Secondly, we posit that ethanol (EtOH), a semi-polar solvent miscible in both the water and oil phases of the W/O₁/O₂/W emulsion, will alter the protein localization/distribution within composite MPs due to multifaceted effects on solvent-solvent, polymer-solvent, and protein-polymer interactions.³⁹ We report achieving distinct release profiles where the delay period and subsequent protein release rates are related to solvent evaporation parameters and EtOH content. Thus, the described MPs represent a tunable and easily adoptable toolset for controlling the temporal dynamics of encapsulated protein payloads to ultimately emulate biochemical signalling cascades for a variety of therapeutic applications.

2. Methods and Materials

2.1 Materials

Poly(lactic-*co*-glycolic) acid (PLGA; 50:50 ester-terminated; inherent viscosity = 0.55–0.75dL/g) was purchased from Lactel (Birmingham, USA). Poly(L-lactic) acid (PLLA; MW = 40–70 kDa) was supplied by Polysciences (Taipei, Taiwan). This particular combination of PLGA and PLLA molecular weights is expected to be immiscible⁴⁰. Dichloromethane (DCM) was acquired from Alfa Aesar (Ward Hill, USA) and dimethyl sulfoxide (DMSO) from American Bioanalytical (Natick, USA). All other materials and chemicals were purchased from Sigma-Aldrich (St. Louis, USA) and used without further modification or purification.

2.2 Fabrication of layered Microparticles (MPs)

Protein-loaded PLGA-PLLA microparticles were fabricated using two protocols, where their solvent evaporation periods and supplementation of excess dichloromethane (DCM) in the aqueous continuous phase are the principal differences (hereinafter referred to as either short or long solvent evaporation groups). The overall fabrication protocol is adapted from previously described techniques (Figure 1).^{14,19} Both PLGA (260 mg/mL) and PLLA (130 mg/mL) were dissolved in DCM, where EtOH (0, 1, 2.5, 4, 5 & 7%; v/v) was added only to the PLLA solution. The aqueous protein phase (BSA in 1× PBS) was added dropwise in the PLGA solution and vortexed for 20 s (total BSA content = 2% (w/w) PLGA). This first emulsion was transferred dropwise to the PLLA solution and the resulting mixture was subsequently added dropwise to a 4× volume excess of 1.0% (w/v) poly-vinyl alcohol (PVA) + 2.5% (w/v) sodium chloride (NaCl) + DCM (0 or 1.6% (v/v))^{14,15,41}. Excess DCM in the continuous phase was hypothesized to reduce DCM flux from the dispersed oil phase and was added only for the long solvent evaporation groups. This second emulsion was vortexed on high settings for 20 s using a tabletop vortexer and transferred to a 15× excess of 0.5% PVA + 2.5% NaCl for solvent evaporation, either for 5 hrs (short solvent evaporation) or overnight (long solvent evaporation). The MPs in the long solvent evaporation groups were allowed to harden overnight to allow for complete precipitation of the polymers. The MPs were recovered with three subsequent washes with deionized (DI) water and lyophilization. Control blank MPs for each group were fabricated under identical conditions without the addition of BSA.

2.3 Characterization of Microparticle Morphology and Polymer Localization

MP size and architecture were evaluated through scanning electron microscopy (SEM; Phillips XL-30; San Francisco, USA) with a 3–5 kV electron beam. Lyophilized particle samples were prepared for SEM analysis via a gold/palladium sputter coater (108-Auto, Cressington Scientific; Watford, UK) to achieve a 5–10 nm thick layer. A minimum of 7 representative images were captured per group and analyzed using ImageJ to determine the MP size distributions (>600 data points/group). MP cross-sections were imaged using SEM after slicing the MPs with a razor blade on a cold surface prior to sputter coating. Polymer localization was visualized by dissolving the PLGA fraction of sliced particles in dimethyl sulfoxide (DMSO; PLLA is insoluble in DMSO) for 1.5 hrs and washing three times with DI water. After lyophilization, the same sputter coating sample preparation procedure was

used to visualize the remaining PLLA fractions via SEM. We completed 4–5 sample preps for each group in order to acquire at least one SEM image of a sliced/dissolved MP per group.

2.4 Protein Localization Analysis

Protein localization was quantified by encapsulating Alexa-568 conjugated-insulin within each particle group (fabricated according to the methods outlined above). Fluorescence and transmitted light images of insulin-loaded MPs for each group were captured using a Leica SP5 laser confocal microscope (Wetzlar, Germany) with a 40× objective. Multiple optical cross sections were imaged for each MP and subsequent quantification of protein localization was performed using MATLAB® (The Mathworks Inc., Natick, USA). A minimum of three optical slices per MP were selected, centered around the middle of each MP. For each of the image slices, the transmitted light images were used to generate twenty lines between the edges of the MPs through its center, ensuring even coverage across the MP cross-sections. Fluorescence intensity across each line was then mapped, and averaged between the selected optical slices for every MP. A minimum of twenty particles was quantified to determine protein localization for each group. The MP diameters were normalized where 0 represents the middle and –1 and +1 correspond to the edges of the MPs. The fluorescence intensity was normalized based on the maximum value for each MP.

2.5 Protein Loading Assays

Total protein loading was determined by complete dissolution of MPs in DCM (10 mg/mL) followed by multiple protein extraction steps.^{17,20} Briefly, the DCM solution containing dissolved MPs was diluted 1:2 in 2.5% (w/v) sodium dodecyl sulphate and 0.1 N NaOH in DI water and thoroughly agitated for 1 hr at 37 °C. The mixture was then centrifuged to separate the aqueous/organic phases and the aqueous phase was removed and replenished. This solvent extraction process was repeated four times, at which point the protein content extracted in the aqueous phase was determined using a bicinchoninic assay (BCA; G Biosciences; St. Louis, USA). Blank MPs (no BSA loaded) were subjected to the same process and served as negative controls (n = 3 per group). The data are presented as loading capacity; BSA content (µg) per mass of MPs (mg).

2.6 Protein Release Assays

For the release assays, 10 mg of lyophilized particles were resuspended in 1 mL of release media (1× PBS supplemented with 0.01% Tween 80 and 0.01% NaN₃) and incubated under constant agitation at 37 °C (n = 3 per group). At specified time points (1, 6, 12, 24, 48 hours; every 2 days until day 14; every 4 days until day 58) the supernatant was sampled by centrifuging the particle suspension at 10,000g for 15 mins to collect 90% of the supernatant and replenishing with fresh release media. Extracted buffer release media samples were stored at –80 °C for subsequent protein analysis with BCA. Release media from blank MPs served as negative controls.

2.6 MP Degradation Studies

Protein-loaded MPs from the short and long solvent evaporation groups were resuspended in buffered release medium (10 mg/mL) and underwent the same regimen as the release assay described above. Upon replacing the release media at 5, 15, 30, and 60 day time points, a portion of the resuspended MPs was extracted and stored at -80°C (following washing with DI water and lyophilization). The degraded MP samples (intact and sliced) were then processed and prepared for SEM analysis using the same protocol described above. Representative micrographs were acquired to determine the morphology of the MP surface and interior.

2.7 Statistics

Statistical analysis was performed on all quantitative assays (GraphPad Prism, La Jolla, CA). All results are depicted as the mean $\pm$ one standard deviation, unless otherwise stated. Statistical analyses evaluated differences between groups using analysis of variance (ANOVA), followed by Tukey post-hoc tests to determine statistical significance, with $p < 0.05$ considered significant. Multiplicity adjusted p -values are reported for Tukey post-hoc comparisons.

3. Results and Discussion

In this study, we identified two mechanisms to alter encapsulated protein distribution within multi-layered PLGA/PLLA composite MPs and subsequent release profiles. First, we demonstrated that the solvent evaporation/extraction (particle hardening) process of the W/O₁/O₂/W method is critical in determining the localization/distribution of encapsulated protein and thus, the resulting protein release profiles. Secondly, we employed the semi-polar solvent, EtOH in the W/O₁/O₂/W process to increase the solubility of DCM in water,³⁹ thereby significantly affecting the polymeric structures and protein distribution within the MPs. The remaining results/discussion section aims to dissect the effects of these two different methods and explore their proposed mechanisms.

3.1 Particle Hardening Parameters Affect Layered MP Structure

Layering/phase separation of PLGA and PLLA polymers in MPs is attributed to differences in hydrophilicity and polymer crystallinity (PLLA is semi-crystalline; PLGA is amorphous and relatively more hydrophilic). These two properties facilitate phase separation between the polymers creating PLGA-rich and PLLA-rich layers during solvent evaporation.^{38,42} The final polymer configuration of the hardened MPs depends on hardening time and rate of polymer precipitation.⁴² A slow rate of polymer precipitation and a long hardening time (long solvent evaporation group) favor the thermodynamic equilibrium orientation of the PLGA and PLLA polymers dictated by conditions such as surface and interfacial tensions.^{12,13,38} On the other hand, a fast solvent efflux and a short hardening time (short solvent evaporation group) will yield a structure determined more by kinetic factors. Here, a fast polymer precipitation rate limits mobility of the polymer chains that may impede the adoption of the thermodynamically favored PLGA/PLLA orientation.^{38,43} Thus, we posit that the MPs in the short/long solvent evaporation groups would result in differing MP

structures and protein distribution, dependent on the solvent efflux rate as well as hardening time.

SEM micrographs of representative particles from the short and long solvent evaporation groups demonstrated the production of spherical composite MPs (Figure 2) with complex layered architectures within each MP suggesting successful phase separation between the PLLA and PLGA (Figure 2C & G). The short solvent evaporation group exhibited a single core-shell architecture (Figure 2C), whereas the long solvent evaporation groups had a more prominent multicore-shell (multicore) structure (Figure 2G).⁴⁴ Interestingly, the multicore structures achieved with the long solvent evaporation seemed to aggregate in close proximity to the MP center as well as at the edges of the MPs (Figure 2G). The average MP diameters were significantly different ($p < 0.01$) at $32.8 \pm 17.4 \mu\text{m}$ and $80.0 \pm 32.5 \mu\text{m}$ for the short and long solvent evaporation groups, respectively. It is generally expected that slower solvent evaporation over a longer period of time leads to a gradual and more complete precipitation of the polymer(s), thus resulting in relatively non-porous particles with a smaller average diameter.^{8,45} However, we observed the opposite effect, where the long solvent evaporation group produced MPs with larger average diameters. The excess DCM in the continuous phase likely allowed the dispersed oil phase in the long solvent evaporation group to remain solvated for a longer period of time. Thus, we speculate that coalescence and agglomeration of the oil droplets during solvent evaporation may contribute to the increase in the average MP diameter.⁴⁵

Lateral and stratified movement of polymer chains are two major types of phase separation thought to occur during solvent extraction in immiscible polymer blends.^{23,43,46} Solvent flux is highest in a small volume at the outermost edge of the oil droplets where the polymer constituents precipitate at a faster rate relative to the core.^{23,46} High rate of polymer precipitation favors lateral phase separation of polymer constituents, which often results in a rough surface morphology.⁴³ Whereas, stratified phase separation may still occur in more solvated regions of the MP interior, leading to a core-shell architecture.^{43,46} In this study, the short solvent evaporation process produced MPs that exhibited signs of both types of PLGA/PLLA phase separation; namely, rough surface morphology (lateral separation; Figure 2B) and a distinct core phase (stratified separation; Figure 2C). In contrast, the long solvent evaporation led to MPs with a relatively smooth surface morphology (Figure 2F) with evidence of stratified phase separation near the MP core (Figure 2G). To probe the polymeric distribution, MPs were sliced and exposed to DMSO (dissolves only PLGA) then imaged with SEM to assess the remaining PLLA-rich structure. The MPs in short and long evaporation groups contained evidence of PLLA shell structures, suggesting that PLGA largely comprised the core fraction for both groups (Figure 2D & H), consistent with previous studies.^{11,19} Interestingly, the PLLA fraction for the long solvent evaporation MPs exhibited a more porous structure compared to the non-DMSO treated MPs (Figure 2G vs H). This observation further supports the speculation that the long solvent evaporation method resulted in a central PLGA-rich core engulfed by a PLLA-rich region, impregnated with aggregates of PLGA leading to a multicore architecture. After DMSO treatment, the PLLA-rich portion of the long solvent evaporation group failed to retain evidence of the thin, outer-most layer (Figure 2G) with a high concentration of phase-separated spheres. This observation suggests that the edges of the long solvent evaporation MPs are composed

largely of PLGA and are likely to be PLGA fractions of coalesced oil droplets during solvent evaporation (Figure 2G). In summary, the short solvent evaporation process exhibited properties indicative of a single core-shell architecture, whereas the long solvent evaporation exhibited a multicore structure with more complex polymer distribution across the MPs.

3.2 Polymer/Protein Localization & Particle Stability Affect the Protein Release Profile

Emulsion-based methods are frequently used to encapsulate a range of small-molecule hydrophilic drugs^{11,14,15}, as well as a range of proteins.^{20,25,40,47} In this study, solvent evaporation parameters significantly influenced total protein loading for the MPs ($p < 0.01$) with $2.7 \pm 0.4 \mu\text{g}$ and $3.9 \pm 0.4 \mu\text{g}$ BSA/mg particles for the short and long solvent evaporation groups, respectively. Spatial distribution of encapsulated protein was evaluated through confocal microscopy of MPs encapsulated with Alexa-568 conjugated-insulin. The results revealed protein predominantly compartmentalized within the PLGA-rich core of the MPs for the short solvent evaporation group (Figure 3A & C). A similar pattern of polymer and protein distribution was also reported by Rahman *et al.* for layered PLGA/PLLA MPs fabricated with comparable protocols.⁴⁰ In contrast, the long solvent evaporation groups exhibited a more complex pattern of protein distribution. Specifically, large amounts of protein were detected at the edges and the central core of the MPs, whereas the areas in between were relatively protein-depleted (Figure 3B & C). Water soluble protein cargo (in this case, Alexa-568 conjugated-insulin or BSA) is expected to preferentially complex with PLGA-rich regions due to its relative hydrophilicity and amorphous nature compared to PLLA.¹⁵ As such, the patterns in protein localization matched the distribution of PLGA as deduced from the DMSO-treated MPs of both the short and long solvent evaporation groups (Figures 2 & 3).

Differences in polymer/protein localization between the short and long solvent evaporation groups significantly influenced their overall release profiles, specifically the protein release rates. The delay period following the initial burst phase was estimated to be ~4 days vs. ~14 days for the short and long solvent evaporation groups, respectively (Figure 3D). Forty five days into the release assay, 33.7% vs. 87.9% of the encapsulated protein was released from the short and long solvent evaporation groups, respectively (Figure 3D). However, the burst release (cumulative release in the first day) was relatively unchanged at 13.6% vs. 14.1% for the short and long solvent evaporation groups, respectively. Layered MPs with cargo localized in a fully engulfed core, generally exhibit a low burst release (<20% cumulative burst release) as only a small portion of the cargo is available to participate in the burst phenomena.^{1,19,40} As such, previous studies report high burst release when hydrophilic cargo is localized near the shell phase.^{15,40} It was thus surprising that both the short and long solvent evaporation groups had similar burst phenomena, given the differences in their protein distribution (Figure 3A & B). Despite the proximity of the encapsulated protein to the edge of the long solvent evaporation MPs, its release is likely still limited by erosion of PLGA and PLLA rather than simple diffusion to reach the release media.

3.3 Putative Release Mechanisms

After the first 24 hrs, the release profile is determined by a combination of polymer hydrolysis, erosion, subsequent development of pore-network formation, and finally, diffusion of the protein payload¹. We tracked MP polymer erosion visually through SEM at select time points for both long and short solvent evaporation groups to gain insight into possible release mechanisms (Figure 4). For the short evaporation groups, only ~15% of the cumulative cargo was released within the first 5 days. SEM analysis revealed the emergence of small pores on the MP surface (Figure 4A) with limited evidence of bulk polymeric erosion (i.e. cross-sectional images indicated intact particle structures; Figure 4D), thereby impeding protein release. By day 15, another ~12% cumulative cargo was released, suggesting the formation of suitable pore networks mediating slow protein release; qualitative evidence of such a pore network was observed through SEM (Figure 4B & E). Subsequent protein release continued at a slow rate until day 58, where only 36.4% of the cumulative cargo was released (Figure 3D). SEM micrographs indicate complete erosion of the PLGA-rich core and a porous network architecture on the PLLA-rich shell, patterns that are comparable to previous studies (Figure 4F).^{11,17,25} However, such low cumulative release may be an indicator of BSA instability within the short solvent evaporation MPs. Protein denaturation, peptide bond hydrolysis, noncovalent aggregation and protein adsorption to the polymer surface are known factors in long release from PLGA matrices that may affect measured protein release profiles.^{2,48} Therefore, bioactivity assessment of encapsulated protein must be conducted for future controlled release systems designed for specific applications.^{2,48}

Initially, the long solvent evaporation group mirrored the short evaporation group with evidence of pore formation on the surface on day 5 and ~15% of cumulative cargo release (Figures 3D & 4G). The porous surface morphology was also present on day 15, again with limited signs of polymeric bulk erosion (Figure 4H & K). Despite high protein localization at the edges of the MPs, only ~4% of cumulative cargo was released for the long solvent evaporation group between days 5 and 15, relative to the short solvent evaporation group (~12% of cumulative cargo in the same time frame; Figure 3C & D). Slow protein release is likely due to complete entrapment of protein-rich PLGA phases by PLLA and decreased initial porosity of the long solvent evaporation MPs (Figure 2G). By day 30, we observed significant polymer erosion, which was particularly apparent near the surface (Figure 4L). PLLA degrades at a significantly slower rate than PLGA (24 months versus 1–3 months); thus, the heterogeneity and substantial erosion of the MP surface layer further suggests high PLGA content.^{42,49,50} Degradation and collapse of the PLGA-rich sections correlate with the significant increase in protein release rate within this time frame (Figure 3D). In addition to surface polymer erosion, particle debris was also prevalent throughout the long solvent evaporation 30-day samples. This observation suggests that MP disintegration and polymer/protein localization are key determinants of the protein release profile of MPs prepared with a long solvent evaporation.

3.4 EtOH Affects Protein Localization, MP Surface Porosity & Protein Release Profiles

The polarity of a solvent (represented by its dielectric constant, ϵ) plays a significant role in determining its miscibility with other solvents. DCM, a nonpolar solvent ($\epsilon = 8.9$), is

immiscible with polar solvents such as water ($\epsilon = 80.1$). In contrast, semi-polar solvents such as ethanol (EtOH, $\epsilon = 24.5$) are miscible with both water and DCM and act to reduce the dielectric constant gradient between DCM and water, thereby increasing the solubility of DCM in water.³⁹ To the best of our knowledge, we are the first to investigate the effects of supplementing EtOH in the W/O₁/O₂/W process for layered MPs. A more favorable interaction between the polymer solvent and non-solvent is expected to modulate both the thermodynamic and kinetic factors that are crucial in determining the properties of layered MPs.^{12,40} Specifically, EtOH may 1) affect DCM-water flux in/out of the oil droplets during solvent evaporation, similar to a more water-miscible solvent (i.e. DMSO); 2) modulate interactions between the internal aqueous phase (protein solution during MP fabrication), continuous aqueous phase, and the DCM-rich oil droplets; 3) lead to differential polymer and protein distribution within the hardened MPs that ultimately alters the protein release profile. The effect of EtOH was tested in a dose-dependent manner in both the short and long solvent evaporation groups to elucidate its role as a novel means of tuning release profiles from layered MPs.

3.5 EtOH with Short Solvent Evaporation

Upon addition of EtOH during short solvent evaporation, the average particle size for EtOH groups was significantly larger ($p < 0.01$; ranging between 44.3–73.36 μm), compared to the control MPs (0% EtOH; Table 1). Total protein loading was generally higher for the EtOH groups, varying between 2.7–5.3 $\mu\text{g BSA/mg MPs}$, where the 1 & 7% EtOH groups were significantly higher ($p < 0.01$) compared to 0% EtOH (Table 1). SEM micrographs indicate that EtOH MPs exhibited increased surface porosity (Figures 5A, D and supplementary figure 1; S1). Protein localization within the EtOH MPs also differed compared to the control, where a significant portion of the encapsulated protein was detected outside of the central core. Protein distribution was increasingly more diffuse, relative to EtOH content (Figures 5, 6A & S1). All EtOH groups had triphasic release profiles composed of a burst, lag (delay) and fast release phases in contrast to the 0% EtOH control, which lacked a fast release phase within the timeframe allotted for the release assay (Figure 6B). The magnitude of the burst release (14.8–22.4%) was directly proportional to EtOH content between the 1–5% EtOH groups. Additionally, EtOH content modulated the delay period by a factor of 5 (between ~4 and ~22 days) and was inversely proportional to EtOH supplement. However, the 7% EtOH group was an exception to both of these trends, exhibiting the lowest burst release (10.0% cumulative release), as well as the longest delay period of (~26 days) of all the EtOH short solvent evaporation groups.

Previous studies have reported higher surface/bulk porosity for PLGA devices fabricated using solvents with high miscibility in water (i.e. DMSO).^{51,52} EtOH-mediated increase in water/DCM interaction may cause 1) enhanced water penetration from the aqueous continuous phase into the dispersed oil phase, and 2) faster movement of DCM into the continuous phase, increasing the rate of polymer precipitation. EtOH groups thus exhibited alterations in MP structure (diameter & porosity), greater protein loading and a more diffuse protein distribution. As a result, the protein release rate for all EtOH groups was significantly faster compared to the control short solvent evaporation group that released ~36% of cumulative cargo after 58 days (Figure 6B). More specifically, EtOH affected both

the burst (directly proportional to EtOH content), and the delay period (inversely proportional to EtOH content). The 7% EtOH group, however, did not follow these trends. The 7% group also exhibited a unique protein distribution pattern with a lower amount of protein localized in the center of the MPs (Figures 6A and S1) compared to the 0–5% EtOH groups. The exact mechanism behind a possible trend reversal in release profile, and its relationship with protein observed distribution for the 7% EtOH group is unclear. Further studies are required to determine how high amounts of EtOH may differentially affect various interactions (between polymer(s), cargo, solvent and non-solvent), and their interplay in the W/O₁/O₂/W process.³⁹ The data suggest that EtOH in the short solvent evaporation groups altered both MP structural properties (i.e. diameter and possibly porosity), as well as protein distribution within the MPs (Figure 9). Protein distribution across the MPs was more diffuse, whereas increased average diameters, prominent surface porosity and fast protein release of the EtOH groups suggest the formation of porous networks within the interior of the MPs (Figure 9A & C).

3.6 EtOH with Long Solvent Evaporation

The addition of EtOH in the long solvent evaporation method generally decreased the average particle size compared to the 0% EtOH control with the exception of the 2.5% EtOH (Table 2). However, EtOH supplement did not affect the total protein loading (Table 2). SEM analysis revealed the emergence of pores on the surface of the MPs with the addition of EtOH (Figures 7 & S2). Protein localization patterns indicated high protein content encapsulated either near the central core or the edges of the MPs, regardless of EtOH content (Figures 7, 8A & S2). However, we did note a prominent increase in protein levels in the intermediate, PLLA-rich region of the EtOH MPs (particularly the 4 & 7% EtOH groups), resulting in a relatively more homogenous protein distribution compared to the 0% EtOH control (Figure 8A). The overall release profiles were triphasic (initial burst, lag phase and fast subsequent protein release) for all long solvent evaporation groups. EtOH in this case largely affected the lag phase (delay period) and the burst release (Figure 8B). The delay period was directly proportional to the EtOH content and ranged between 18 and 30 days. Conversely, the amount of burst release trended lower with respect to EtOH content (Figure 8B).

The oil droplets in the long solvent evaporation groups are expected to stay solvated for a longer period of time, allowing the polymer chains and the protein payload to organize closer to their thermodynamic equilibrium.^{12,38} This mechanism may explain the lack of change in protein loading, and overall shapes of the release profiles between the EtOH and control MPs (Figure 8). Additionally, the smaller diameter and longer delay periods in the protein release profiles, as well as less prevalent surface pores (compared to short solvent evaporation EtOH groups) suggest that water penetration into the dispersed oil phase is likely not as important a parameter in the long solvent evaporations EtOH groups. EtOH-mediated protein localization within the intermediate PLLA-rich layer translated to a smaller fraction of the encapsulated protein available for the burst release, reflected in a decreased burst release (Figure 8B). Furthermore, the EtOH groups also exhibited longer delay periods, likely due to greater protein (and/or PLGA) localization within the PLLA-rich intermediate layer, rather than the PLGA-rich outermost layer. To summarize, EtOH in the

long solvent evaporation groups altered protein release profiles largely by modulating protein (and possibly polymer) localization within the MPs (Figure 9). EtOH groups exhibited a more diffuse protein distribution across the MPs; whereas, their lower average diameters and longer delay periods suggest that pores visible in the SEM micrographs are likely to be limited to the surface and regions close to the surface (Figures 9C & D).

4. Conclusion

W/O₁/O₂/W techniques were used to fabricate protein-loaded layered MPs where the solvent evaporation parameters (time and solvent flux) affected particle morphology (average diameter and protein/polymer distribution), which led to significant differences in the resulting protein release profiles. The short solvent evaporation group had encapsulated protein compartmentalized largely in the MP core, whereas protein in the long solvent evaporation group was concentrated both near the MP core and its outer edges. After an initial burst, the short solvent evaporation group exhibited slow release of protein throughout the allotted time for the release assay. On the other hand, the long solvent evaporation group had a distinct delay period (~14 days), followed by fast protein release in the subsequent ~25 days. We also demonstrated that EtOH was supplemented (between 0–7%; v/v) in the PLLA solution of the short/long solvent evaporation groups as a novel means of modulating critical protein release characteristics. We hypothesized that the EtOH modulates interactions between solvent/non-solvent phases during solvent evaporation and thus, we demonstrated differential protein distribution in hardened MPs in the EtOH supplemented groups, regardless of solvent evaporation parameters. In addition, EtOH also alters structural properties such as average diameter, porosity and polymer distribution of layered MPs. Alterations in protein distribution and MP structure ultimately enable tuning of the delay period (between 0–30 days after an initial burst release) and total protein release periods (~30 – >58 days) based on solvent evaporation parameters and EtOH content. Further studies are required to determine the exact mechanisms of how EtOH affects the thermodynamic and kinetic factors that determine the formation of layered MPs in the W/O₁/O₂/W process. Additionally, bioactivity of loaded protein also needs to be verified and optimized, as the encapsulation and release processes are known to induce protein aggregation and denaturation. We believe that the described techniques may be adapted and optimized for other proteins of interest and used as a tool in diverse applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Dutta D, Salifu M, Sirianni RW, Stabenfeldt SE. *J Biomed Mater Res A*. 2016; 104:688–696. [PubMed: 26517011]
2. Zhu G, Mallery SR, Schwendeman SP. *Nat Biotechnol*. 2000; 18:52–57. [PubMed: 10625391]
3. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. *J Controlled Release*. 2012; 161:505–522.
4. Moghimi SM, Hunter AC, Murray JC. *Pharmacol Rev*. 2001; 53:283–318. [PubMed: 11356986]
5. Chung TW, Huang YY, Liu YZ. *Int J Pharm*. 2001; 212:161–169. [PubMed: 11165073]
6. Stureson C, Artursson P, Ghaderi R, Johansen K, Mirazimi A, Uhnöo I, Svensson L, Albertsson AC, Carlfors J. *J Controlled Release*. 1999; 59:377–389.
7. Hines DJ, Kaplan DL. *Crit Rev Ther Drug Carrier Syst*. 2013; 30:257–276. [PubMed: 23614648]
8. Freitas S, Merkle HP, Gander B. *J Controlled Release*. 2005; 102:313–332.
9. Bodmeier R, McGinity JW. *Pharm Res*. 1987; 4:465–471. [PubMed: 3508558]
10. Jalil R, Nixon JR. *J Microencapsul*. 1990; 7:53–66. [PubMed: 2308054]
11. Li X, Li L, Wang X, Ren Y, Zhou T, Lu W. *J Pharm Sci*. 2012; 101:3946–3961. [PubMed: 22753275]
12. Pekarek KJ, Jacob JS, Mathiowitz E. *Nature*. 1994; 367:258–260. [PubMed: 8121490]
13. Pekarek KJ, Jacob JS, Mathiowitz E. *Adv Mater*. 1994; 6:684–687.
14. Lee TH, Wang J, Wang CH. *J Controlled Release*. 2002; 83:437–452.
15. Tan EC, Lin R, Wang CH. *J Colloid Interface Sci*. 2005; 291:135–143. [PubMed: 15964579]
16. Tan H, Ye J. *Appl Surf Sci*. 2008; 255:353–356.
17. Xia Y, Ribeiro PF, Pack DW. *J Controlled Release*. 2013; 172:707–714.
18. Xia Y, Xu Q, Wang C, Pack DW. *J Pharm Sci*. 2013; 102:1601–1609. [PubMed: 23529836]
19. Zheng W. *Int J Pharm*. 2009; 374:90–95. [PubMed: 19446764]
20. Ansari RH, Rahman MM, Awang MB, Katas H, Hadi H, Mohamed F, Doolaanea AA, Kamaruzzaman YB. *Arch Pharm Res*. 2016; 39:1242–1256. [PubMed: 26818028]
21. Roy I, Gupta MN. *Biotechnol Appl Biochem*. 2004; 39:165–177. [PubMed: 15032737]
22. Harkins WD. *J Chem Phys*. 1941; 9:552–568.
23. Pollauf EJ, Pack DW. *Biomaterials*. 2006; 27:2898–2906. [PubMed: 16439013]
24. Yuan W, Liu Z. *Int J Nanomedicine*. 2012; 257. [PubMed: 22287838]
25. Kokai LE, Tan H, Jhunjunwala S, Little SR, Frank JW, Marra KG. *J Controlled Release*. 2010; 141:168–176.
26. Xu Q, Chin SE, Wang CH, Pack DW. *Biomaterials*. 2013; 34:3902–3911. [PubMed: 23453059]
27. Finch AR, Caunt CJ, Armstrong SP, McArdle CA. *Am J Physiol - Cell Physiol*. 2009; 297:C591–C600. [PubMed: 19587220]
28. Olanow CW, Obeso JA. *Ann Neurol*. 2000; 47:S167–176–178. [PubMed: 10762145]
29. Overstreet DJ, Dutta D, Stabenfeldt SE, Vernon BL. *J Polym Sci Part B Polym Phys*. 2012; 50:881–903.
30. Imitola J, Raddassi K, Park KI, Mueller FJ, Nieto M, Teng YD, Frenkel D, Li J, Sidman RL, Walsh CA, Snyder EY, Khoury SJ. *Proc Natl Acad Sci U S A*. 2004; 101:18117–18122. [PubMed: 15608062]
31. Itoh T, Satou T, Ishida H, Nishida S, Tsubaki M, Hashimoto S, Ito H. *Neurol Res*. 2009; 31:90–102. [PubMed: 19228460]
32. Dutta D, Fauer C, Mulleneux HL, Stabenfeldt SE. *J Mater Chem B*. 2015; 3:7963–7973.
33. Yi X, Jin G, Zhang X, Mao W, Li H, Qin J, Shi J, Dai K, Zhang F. *PLoS ONE*. 2013; 8:e70306. [PubMed: 23922973]
34. Stabenfeldt, Addington C, Dutta D, Roussas A. *Biomark Insights*. 2015:43.
35. Chu H, Wang Y. *Ther Deliv*. 2012; 3:693–714. [PubMed: 22838066]
36. Awada HK, Johnson NR, Wang Y. *J Control Release Off J Control Release Soc*. 2015; 207:7–17.
37. Zhang L, Lu Z, Zhao Q, Huang J, Shen H, Zhang Z. *Small*. 2011; 7:460–464. [PubMed: 21360803]

38. Leach K, Noh K, Mathiowitz E. *J Microencapsul.* 1999; 16:153–167. [PubMed: 10080110]
39. Luan X, Skupin M, Siepmann J, Bodmeier R. *Int J Pharm.* 2006; 324:168–175. [PubMed: 16854540]
40. Rahman NA, Mathiowitz E. *J Controlled Release.* 2004; 94:163–175.
41. Jiang G, Thanoo BC, DeLuca PP. *Pharm Dev Technol.* 2002; 7:391–399. [PubMed: 12503521]
42. Södergård A, Stolt M. *Prog Polym Sci.* 2002; 27:1123–1163.
43. Cheng D, Cao X, Gao H, Ye X, Li W, Wang Y. *RSC Adv.* 2014; 4:9031.
44. Trojer MA, Nordstierna L, Nordin M, Nydén M, Holmberg K. *Phys Chem Chem Phys.* 2013; 15:17727–17741. [PubMed: 23999918]
45. Sharma N, Madan P, Lin S. *Asian J Pharm Sci.* 2016; 11:404–416.
46. Tang ZG, Rhodes NP, Hunt JA. *Chem Eng Res Des.* 2007; 85:1044–1050.
47. Kokai LE, Ghaznavi AM, Marra KG. *Biomaterials.* 2010; 31:2313–2322. [PubMed: 19969346]
48. Ye M, Kim S, Park K. *J Controlled Release.* 2010; 146:241–260.
49. Middleton JC, Tipton AJ. *Biomaterials.* 2000; 21:2335–2346. [PubMed: 11055281]
50. Schmitt EA, Flanagan DR, Linhardt RJ. *Macromolecules.* 1994; 27:743–748.
51. Park TG, Lee HY, Nam YS. *J Controlled Release.* 1998; 55:181–191.
52. Ahmed TA, Ibrahim HM, Samy AM, Kaseem A, Nutan MTH, Hussain MD. *AAPS Pharm Sci Tech.* 2014; 15:772–780.

Fig. 1.

A modified W/O₁/O₂/W technique was employed to fabricate all layered microparticles in the study. In short, the protein payload dissolved in aqueous buffer was first emulsified with the PLGA phase (dissolved in dichloromethane, DCM). After vortexing the mixture, the W/O₁ emulsion was added to the PLLA phase (dissolved in DCM containing various volume ratios of ethanol, EtOH) dropwise, vortexed and added to a stirring aqueous solution of emulsifier, producing the W/O₁/O₂/W emulsion. DCM was then allowed to evaporate and the precipitated particles were recovered through subsequent washing and lyophilization steps.

Fig. 2.

Population and structural properties of the short (A–D) and long (E–H) solvent evaporation microparticles (MPs). (A and E) Representative scanning electron micrographs (SEM) indicate the formation of spherical MPs for all groups. The short solvent evaporation MPs exhibited a rough surface morphology (B) relative to the long solvent evaporation group (F). Layered MP morphology was observed in the cross-sections of both the short (C) and long (G) solvent evaporation MPs. Selective dissolution of PLGA using DMSO in sliced MPs indicated a PLGA-rich core engulfed in a PLLA-rich layer for both the short (D) and long (H) solvent evaporation groups. All scale bars are 20 μm except panels A and E (100 μm).

Fig. 3.

Protein localization and release profiles for the long and short evaporation groups. (A) Representative confocal image of short solvent evaporation group with protein compartmentalization in the core of the MP. (B) Representative confocal image of the long solvent evaporation group, where addition of DCM in the continuous phase and overnight solvent extraction causes significant fractions of the total encapsulated protein to be concentrated both in the core, and the edges of the particle. (C) Quantification of protein localization with respect to the normalized diameter for short/long solvent evaporation groups. (D) Differences in particle structure and protein localization results in significant differences in the overall release profile; the dashed white line represents the particle edge. Scale bars = 20 μm .

Fig. 4.

SEM micrographs of MP degradation during *in vitro* release assays of short (A–F) and long (G–L) solvent evaporation groups. (A and B) Native and unsliced SEM micrographs for short solvent evaporation MPs indicate pore formation at the surface, and minimal polymer erosion across the interior on day 5. (C and D) Qualitative differences in surface features and interior polymer erosion were not apparent 15 days into the release assay. (E and F) By day 60, the short solvent evaporation MPs retained their spherical architecture, whereas sliced MPs revealed well-defined hollow cores with porous shell structures. The long solvent evaporation MPs followed the same trends on days 5 (G and H) and 15 (I and J), with evidence of pore formation at the MP surface, but no apparent polymer erosion in the MP interiors. (K and L) By day 30 however, the long solvent evaporation MPs exhibited rough surface morphologies and prominent evidence of debris, likely due to disintegration of the outermost MP layer. Scale bar lengths are denoted within each panel.

Fig. 5.

Ethanol (EtOH) affects surface porosity and protein localization within short solvent evaporation MPs. (A) SEM micrographs of MPs without any EtOH exhibited minimal porosity at the surface. (B and C) Merged fluorescence and transmitted light images from confocal microscopy suggest that Alexa-568 conjugated-insulin was compartmentalized largely near the central core of the MPs. (D) Upon addition of 5% (v/v) EtOH, SEM micrographs indicate increased surface porosity. (E and F) Confocal images indicate a more diffuse protein distribution in the 5% (v/v) EtOH group; the dashed white line represents the particle edge. Scale bars = 50 μm .

Fig. 6.

MP structure and protein localization in the short solvent evaporation MPs affect the protein release profile. (A) Protein localization was quantified with respect to the normalized MP diameter ("0.0" represents the center of the particle; "+/-1.0" are the outer edges) and fluorescence intensity. Addition of EtOH results in a more diffuse protein distribution in an EtOH dose-dependent manner. (B) Cumulative BSA release profile of the short solvent evaporation groups. All EtOH groups exhibited triphasic protein release profiles, whereas the control 0% EtOH group did not exhibit the final fast release phase in the allotted time period of the release study.

Fig. 7.

Ethanol (EtOH) affects protein localization within the long solvent evaporation MPs. (A) SEM micrographs of control (0% EtOH) MPs exhibited little to no surface porosity. (B and C) Merged images of fluorescent and transmitted light suggested Alexa-568 conjugated-insulin was compartmentalized largely near the central core, as well as the edges of the control MPs. (D) Upon addition of 4% (v/v) EtOH, SEM micrographs indicated some increase in surface porosity. (E & F) Confocal images indicated a more diffuse protein distribution in the 4% (v/v) EtOH group where the areas between the central MP core and its edges have more signal relative to control MPs. The dashed white line represents the particle edge. Scale bars = 50 μm .

Fig. 8.

Protein localization in the long solvent evaporation MPs affects the protein release profile. (A) Protein localization was quantified with respect to normalized MP diameter and fluorescence intensity. EtOH groups, especially the 4% and 7% EtOH (v/v) groups, resulted in a more uniform edge-to-edge protein distribution relative to the control. (B) Cumulative BSA release studies from all long solvent evaporation groups exhibited triphasic release profiles. The duration of the intermediate lag phase (delay period) varied between ~14 – 30 days, depending on the EtOH content.

Fig. 9.

Putative effects on the structure of layered MPs with EtOH in the W/O₁/O₂/W process. (A) Protein localization is largely in the PLGA-rich central core of the layered MPs in the 0% EtOH short solvent evaporation group. (B) Upon addition of EtOH in the short solvent evaporation group, protein distribution became more diffuse across the MPs. The increased average diameters, the prominent surface porosity and fast protein release of the EtOH MPs are indicators of the porous network within the MP interior, as indicated by the blue arrow. (C) MPs in the 0% EtOH long solvent evaporation group had protein encapsulated largely in the central core, as well as the outer edges. (D) EtOH groups also exhibited a more diffuse protein distribution across MPs. A decrease in the average diameters of the EtOH MPs, and longer delay periods suggest EtOH supplementation may not lead to the formation of an extensive pore network in the interior of the long solvent evaporation MPs. Instead, observed pores in the long solvent evaporation EtOH groups may be limited to the surface and regions close to the surface, as indicated by the blue arrow.

Table 1

Size and Loading of Short Solvent Evaporation Groups

EtOH Group	Diameter (Avg $\pm$ SD)	Loading (μ g BSA/mg MPs; Avg $\pm$ SD)
0	32.9 $\pm$ 17.4	2.7 $\pm$ 0.4
1	80.7 $\pm$ 19.5 *	4.8 $\pm$ 0.4 *
2.5	62.2 $\pm$ 22.7 *	2.7 $\pm$ 0.9
5	44.3 $\pm$ 15.2 *	3.7 $\pm$ 0.5
7	73.4 $\pm$ 27.6 *	5.3 $\pm$ 0.5 *

* - p<0.01 compared to 0% EtOH

Table 2

Size and Loading of Long Solvent Evaporation Groups

EtOH Group	Diameter (Avg $\pm$ SD)	Loading (μ g BSA/mg MPs; Avg $\pm$ SD)
0	80.0 $\pm$ 32.5	3.9 $\pm$ 0.4
1	62.4 $\pm$ 40.0 *	3.2 $\pm$ 0.2
2.5	76.2 $\pm$ 34.6	3.9 $\pm$ 0.6
4	66.0 $\pm$ 35.5 *	3.8 $\pm$ 0.3
7	68.2 $\pm$ 38.3 *	3.5 $\pm$ 0.2

*
- p<0.01 compared to 0% EtOH