POLYMER ENCAPSULATED DNA VACCINE FOR SUSTAINED RELEASE

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ABSTRACT

POLYMER ENCAPSULATED DNA VACCINE FOR SUSTAINED RELEASE

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To improve the efficacy of single-dose vaccines, we have developed stabilizer-free poly lactic-co-glycolic acid (PLGA) nanoparticle formulations for use as drug and vaccine delivery systems capable of sustained-release. By removing commonly used toxic stabilizers such as poly vinyl alcohol (PVA), we were able to increase cellular viability, while maintaining stability of PLGA nanoparticles through optimization of PLGA composition. Nanoparticle stability is important to shelf life, vaccine encapsulation, and extended release for persistent immune activation. By altering charge, formulation temperature and protein addition, we have developed PLGA nanoparticles with tunable degradation over a one-to-two-month time periods. These improvements in nanoparticle delivery systems should provide for increased efficacy of single-dose vaccines by mimicking the prime-boost schedule currently used.

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CHAPTER 1: INTRODUCTION

Vaccines are designed to evoke an adaptive immune response leading to the recognition and rapid isolation of new infectious agents. Vaccine agents are categorized into, live/attenuated or inactivated organisms, subunit proteins, or genetically encoded (mRNA or DNA) pathogen specific sequences. While vaccines are currently the most efficient way to prevent the spread of infectious disease, there are several disadvantages to current formulations, including (i) inefficient immune response, (ii) need for booster shots and (iii) short product shelf life requiring low temperature storage (Hasson et al., 2015). Current disadvantages stem from inefficient uptake and vaccine instability, resulting in the need for long-lived vaccine delivery systems.

To transport vaccine agents in a safe, effective, and biocompatible manner delivery systems must be capable of encapsulating agents, be taken up by immune cells, and enable release of cargo for cell processing. Additionally, particles capable of controlled vaccine release are designed to eliminate the need for booster shots by consistently exposing your body to the vaccine over an extended period, during which time the body can begin to mount the appropriate immune response.

DNA vaccines introduce a genetically engineered plasmid containing pathogen specific DNA sequences that trigger immune response following transcription and translation of the DNA sequence. Advantages of DNA vaccines include stimulating both B- and T- cell response, improving vaccine shelf life, negating use of an infectious agent, and ease of large-scale manufacturing (Flingai et al., 2013; Hobernik & Bros, 2018; Khan, 2013). These genetic vaccines are relatively new and there are currently no FDA approved DNA vaccines for human use, nevertheless some DNA-based vaccines are FDA and USDA approved for veterinary use (Hobernik & Bros, 2018; Jazayeri & Poh, 2019; Redding & Weiner, 2009). These vaccines are used for West Nile Virus in horses or melanoma in canines. However, there are several DNA based vaccine clinical trials currently being evaluated for use in humans for the treatment of HIV and

Hepatitis B, and thus improvements in their packaging and delivery are important (Corbett et al., 2020; Ledgerwood et al., 2011; Schleiss, 2009).

While promising, DNA vaccines have historically been plagued by low immunogenicity stemming from their need to be first taken up by antigen presenting cells, then released from endosome, translocated to the nucleus for transcription, and finally made into protein before immune recognition can occur. The use of priming and booster shots addresses this issue by providing time between each dose to ramp up the immune response; however, the use of a prime and boost is a significant disadvantage (see below). To improve the transfection efficiency, delivery systems are crucial to constructing a successful DNA vaccine. If designed appropriately, the delivery systems will prevent extracellular DNA degradation, increase DNA stability, and enable sustained release of the vaccine to antigen presenting cells where they can be taken up and the immune system can be efficiently activated without a boost.

There are several FDA approved drugs and vaccines, such as Moderna and Pfizer COVID-19 vaccine, that require the use of delivery systems [Patent US 10,703,789/Patent 10,702,600/US 10,576,146/US 10,485,884]. As these delivery systems do not allow for sustained release multiple shots are necessary to produce sufficient immunization (e.g prime and boost). However, numerous trips to the doctors are inconvenient and can lead to a reduction in protection if second dose are not administered (Nelson et al., 2009). A single-dose vaccine will allow for gradual delivery which may exceed the efficacy observed for multiple injections. This sustained release, or single shot approach, can allow for the secondary boost to be administered gradually without the need for a second doctors visit which saves time and can improve compliance.

While there is little understanding of how to deliver DNA vaccines, lessons can be learned from the field of drug delivery. Some common ways to achieve sustained release for drugs is

through microparticle encapsulation. Spansule, Lupron Depot, and Risperidone are a few of the drugs that use microparticle delivery system formulations for sustained release (Gelvin et al., 1953; Hodgetts et al., 2010; Su et al., 2011; Takada et al., 2003). Currently no vaccines use delivery systems for sustained release, and many are not in the nanoparticle range. Typically administered by injection either intramuscular, intradermal, or subcutaneous, vaccines must be taken up by antigen presenting cells to reach the lymph nodes and produce the desired immune response. The size and charge of delivery systems can significantly affect cellular uptake, cargo release and patient comfort. We will discuss this greatly in the following chapters.

A common polymer used for drug delivery particles, enabling high tunability in particle size from the nano to the micron scale, is poly(lactide-co-glycolide) (PLGA). PLGA is copolymer made of different ratios of, lactic and glycolic acid, that allow for altering the composition of particles. As a Food and Drug Administration (FDA) approved biocompatible polymer, PLGA has been used since the 1990's for microparticle sustained release (Lee et al., 2019). PLGA undergoes ester hydrolysis in the body to produce biodegradable metabolite monomers such as lactic acid and glycolic acid which can be biologically disposed through several physiological and biochemical pathways.

Formulation methods to produce PLGA particles consist of emulsion, solvent evaporation, organic phase separation, microfluidics, and electrospray; with resulting particle sizes ranging from the nano to micro-scale. Due to its low cost and ease of fabrication, solvent evaporation is one of the most frequently used techniques for making PLGA particles. This process is done by dissolving PLGA in an organic solvent and adding the solution dropwise to an aqueous environment. Gradually through solvent evaporation nanoparticles will re-organize and condense

to form stable particles in the aqueous solution. This method allows for flexible incorporation of drug or vaccine components within the various organic or aqueous environments.

The primary drawbacks in PLGA nanoparticle formulation are the large bath-to-batch variations in size and aggregation, leading to inconsistent biological responses. Investigators typically use polymeric stabilizers to lessen these problems. The most common stabilizer for PLGA is polyvinyl alcohol (PVA) (Stromberg et al., 2021; Wang & Walther, 2015). Unfortunately, with the addition of PVA, the particles are cytotoxic and require wash steps to remove PVA from the particles surface. This removal is an inconsistent process that leads to high variability in the sample. In chapter 2 we will be discussing how to make consistent sized particles without the need of stabilizers.

Along with adding stabilizers to create monodisperse and consistent particle formulations, altering the PLGA (e.g. polymer molecular weight, L:G ratio, and charge/size of particle) can alter the particle stability. Each PLGA formulation can achieve different outcomes in terms of degradation and vaccine release profiles, as discussed in chapter 3. One physicochemical characteristic that is important for this loading efficiency and release profile is the surface charge. Additionally, entrapping the vaccine within the PLGA polymer to protect pDNA from nuclease degradation and ensure the integrity, while targeting and sustained release can be accomplished.

Beyond surface charge of delivery systems, particle size can also tune the stability and uptake of these systems. In the micron scale the size of delivery system has a direct correlation to the degradation rate and stability of the particle. Stability at the nanoparticle size range is less known, which is a desirable size regime for vaccine carriers for uptake by antigen presenting cells and not macrophages. Nanosized particles are better than very small delivery systems (<50 nm), which can rapidly move to lymph nodes or larger particles (>500 nm), which are taken up by

macrophages and are poorly immunogenic. Larger sized delivery systems can also lead to pain at injection site and inflammation. In chapter 2 and 3 we will discuss the degradation of these particles regarding size, charge, and pH environments. In chapter 3 we will discuss how we've created a way to consistently produce nanoparticle sizes of 100, 200 and 300 nm with different formulations.

Overall, the current literature on PLGA delivery systems has focused primarily on microparticles and neglecting the implication of these systems within the body's environment for sustained release. The knowledge of reproducible PLGA nanoparticles for vaccine carriers with sustained release is rather limited. Our method will focus on the benefits of nanoparticles, in comparison with microparticles, for IM injection, cell targeting and sustained release. In the following chapters we will discuss the state of the art in the field and compare that to our different nanoparticle formulations and controlling cargo release.

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CHAPTER 2: NON-STABILIZED PLGA NANOPARTICLES

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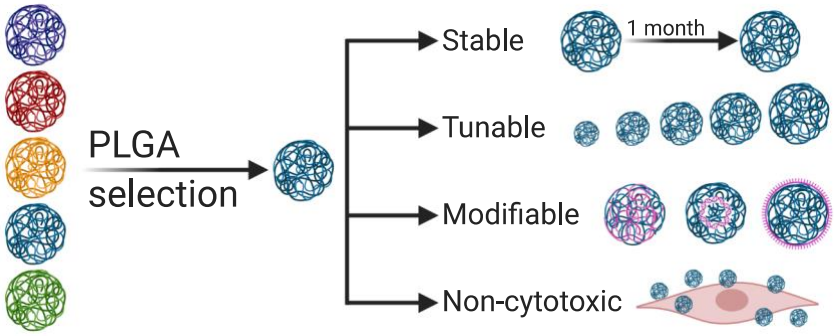


Figure 1. PLGA characteristics necessary for sustained release NPs.

INTRODUCTION

Nanomedicine has proven effective in delivery of therapeutic agents targeting major diseases such as cancer and diabetes, and pathological processes including inflammation, vaccination, and microbial infection (Allahyari and Mohit, 2016; Haggag et al., 2016; Mahmoud et al., 2019; Patra et al., 2018; Wu et al., 2017). However, to provide treatment and enhance therapeutic efficacy, drug and vaccine delivery systems must transport an active agent in a stable, safe, and biocompatible manner to the target site at concentrations high enough to produce a therapeutic response. Delivery systems must be highly tailorable, yet overcome hurdles such as toxicity, clearance, and off-target effects (Tibbitt et al., 2016). Nanoparticle formulation and modification can critically control physicochemical properties such as size, polydispersity, and surface charge, each of which can then affect the behavior of these delivery systems; thus, improvements in nanoparticle synthesis can have a significant outcome on therapeutic results.

The copolymer poly(lactic-co-glycolic acid) (PLGA) has gained attention as a nanoparticle delivery system due to its biocompatibility, biodegradability, sustained release, and ability to be modified (D'Mello et al., 2017; Danhier et al., 2012; Makadia and Siegel, 2011). These favorable characteristics have led to approval of PLGA nanoparticles for therapeutic delivery by the United States Food and Drug Administration (FDA) and European Medicine Agency. There are several

methods for preparation of PLGA particles, such as emulsification, solvent evaporation, and organic phase separation, sometimes coupled with microfluidics or electrospray, to create particles with sizes ranging from the nano to the micron scale. Solvent evaporation is one of the most frequently used techniques for making PLGA particles due to the ability to safely incorporate drug or vaccine components without their loss of function, while creating uniform sized particles through scalable and energy efficient means (Kim et al., 2019; Koerner et al., 2019; Rao and Geckeler, 2011; Sah and Sah, 2015; Xu et al., 2017). In addition to safely encapsulating cargo, solvent evaporation was chosen for our study because of its simplicity, scalability, and reproducibility. With different ratios of lactic and glycolic acid, molecular weight, and functionalization, physicochemical properties such as release kinetics and cellular interactions can, in theory, be tuned for drug delivery and immunization. Yet, batch-to-batch variation and aggregation of PLGA nanoparticles has led to inconsistencies in therapeutic response (Jara et al., 2018). Polyvinyl alcohol (PVA) is typically added to stabilize PLGA nanoparticles and avoid aggregation (Gossmann et al., 2015; Kumar et al., 2004; Makadia and Siegel, 2011; Sahoo et al., 2002). Given the toxicity of PVA, residual polymer must be "removed" following PLGA particle formulation (Boury et al., 1995; Paswan and Saini, 2017; Shakesheff et al., 1997). However, despite incorporating cumbersome wash steps such as centrifugation and dialysis, PVA is often incompletely removed. Residual PVA has been reported to form interconnected networks, permanently attached to the nanoparticle surface due to hydrophobic bonding between partially hydrolyzed PVA [poly(vinyl acetate) groups] and PLGA acetyl groups (Boury et al., 1995; Menon et al., 2012; Murakami et al., 1999, Sahoo et al., 2002). This residual PVA is thought to influence drug release profiles due to a more compact polymer matrix or hydrogel barrier (Sahoo et al., 2002). Further, residual PVA can decrease nanoparticle size, shield surface charge, and decrease

hydrophobicity as compared to PVA-free PLGA particles, all of which can diminish cargo release and cellular uptake of nanoparticles (Sahoo et al., 2002). However, of greatest concern is the potential cytotoxic behavior of residual PVA. Moderate toxicity has been observed in human lung epithelial cells when dosed with high concentrations (>1 mg/mL) of PVA-PLGA nanoparticles (Grabowski et al., 2013). PVA in nanoparticles can also decrease mitochondrial activity and increase reactive oxygen species leading to genotoxicity and inflammation (Grabowski et al., 2015). Furthermore, formulation of PLGA nanoparticles stabilized by PVA nearly always requires sonication, which can be destructive to biological cargo (Grabski, 2009; Wang et al., 2014). While it is generally appreciated that PVA-stabilized PLGA particles may not be ideal, comparably less is understood on how to formulate tunable, stable PVA-free PLGA nanoparticles that are less toxic, but equally as efficacious as PVA-stabilized delivery systems.

While new formulation methods are needed for PVA-free PLGA nanoparticles, as we describe here, surface modification of PLGA offers an additional approach to tune nanoparticle behavior and cellular interaction. Elastin like polymers (ELPs) are proteins composed of repeating amino acid sequences known for their adaptability and biocompatibility (Fazelinia et al., 2017; Ghosh et al., 2018; Lillo et al., 2018; Simon et al., 2017; Varanko et al., 2020). The particular ELP amino acid sequence, as well as the number of repeating units, allows for the tunability of polymer behavior and has been shown to reduce cellular toxicity and enhance drug delivery by altering the release dynamics and biocompatibility of vaccines (Bidwell III et al., 2013). While ELPs have been used previously for increased cellular adhesion and differentiation as tissue scaffolds (Choi et al., 2013) and as vaccine carriers, to date, ELP has not been used in conjunction with PLGA as a delivery system in order to control particle stability and cellular interaction. Specifically, designing an ELP which can effectively be incorporated with PLGA nanoparticles for improved

cellular recognition, while avoiding complications such as coacervation and degradation will be important in creating hybrid polymer nanoparticle drug and vaccine delivery systems.

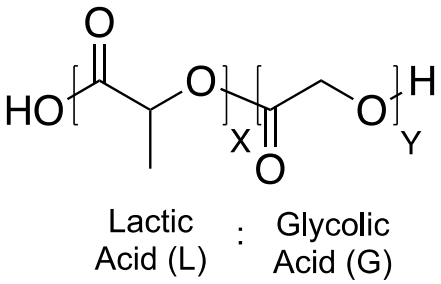
In order to increase particle size uniformity, yield, industrial scalability, and limit the use of toxic solvents and stabilizers, we have developed stabilizer-free PLGA and ELP-PLGA nanoparticles, and their methods, for use as a drug and vaccine delivery systems. A suite of PLGA nanoparticles were synthesized and compared in terms of their physicochemical characteristics with and without incorporation of vaccine plasmid DNA. We examined whether PLGA alone, without PVA, would allow for less aggregation and a consistent size per batch or whether cytotoxic stabilizers were necessary. We then modified PLGA with ELP in order to create stable, yet tunable, particles that are primed for cellular targeting and additional immune tolerance for drug and vaccine delivery systems.

MATERIALS AND METHODS

Design of PLGA-based nanoparticles and solvent evaporation method

PVA-free PLGA nanoparticles were prepared by the solvent evaporation method. Various lactic:glycolic acid ratios, molecular weights, and chemical functionality were examined for their ability to form stable, monodisperse nanoparticles in solution. The following PLGA polymers were obtained from Sigma Aldrich with lactic:glycolic acid ratios, molecular weights, and with carboxylic or ester termination (Fig. 2): P1 polymer (50:50, 40-75 kDa, carboxylic terminated); P2 polymer (50:50, 30-60 kDa, ester terminated); P3 polymer (65:35, 40-75 kDa, ester terminated); P4 polymer (65:35, 24-38 kDa, carboxylic terminated); and P5 polymer (50:50, 24-38 kDa, ester terminated). PLGA synthesis was performed by dissolving 12.5 to 200 mg of polymer in 2.5 mL acetonitrile, followed by dropwise addition of the polymer solution into 7.5 mL (0.2 μm) filtered nanopure water while stirring at room temperature. The PLGA reactions were allowed to proceed

for 24 h while stirring to evaporate the organic phase, then dialyzed overnight against 2 L of nanopure water (with changes of nanopure water after 4 and 16 h) in a 20,000 molecular weight cutoff Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific). Each of the prepared PLGA polymeric nanoparticles (P1-P5) were examined for size and stability in both water and phosphate buffered saline (PBS) by dynamic light scattering (DLS). Given the uniform size and stability of the nanoparticles made with P4 polymer, it was subsequently used as the primary formulation of interest for DNA incorporation, stability, and surface modification.



Polymer	L:G Ratio	Molecular Weight (kDa)	Terminal Group
P1	50:50	40-75	Carboxylic acid
P2	50:50	30-60	Ester
P3	65:35	40-75	Ester
P4	65:35	24-38	Carboxylic acid
P5	50:50	24-38	Ester

Figure 2. Chemical structure of PLGA polymers (P1 – P5) with varying lactic acid to glycolic acid (L:G) ratios, molecular weights, and terminal groups. The number of lactic acid repeats is represented by an "x" and glycolic acid repeats are indicated by a "y".

PVA-PLGA emulsion method

In order to assess the need for surfactants such as PVA in PLGA nanoparticle assembly and stability, PVA-P4 nanoparticles were made using the same solvent evaporation process, as above, PVA was included within aqueous phase, as previously described (Astete and Sabliov, 2006) by emulsion methods. PVA was obtained from Sigma Aldrich (MW 13-23 kDa). For the

emulsion method, P4 was dissolved in 2.5 mL of dichloromethane, and PVA (1-4% wt/vol) was dissolved in 5 mL of nanopure water. P4 was emulsified with PVA on ice using a probe sonicator (Sonic Dismembrator, Model 500, Fisher Scientific) at 50% amplitude with pulsing 1 s on/off for 5 min. After sonication, 15 mL of nanopure water was added to dilute the organic phase and dichloromethane was evaporated overnight on an orbital shaker (200 rpm) at room temperature. To remove PVA, nanoparticles were centrifuged at 17,000 × g for 10 min, the supernatant was discarded and nanoparticles were resuspended in water as previously described (Yang et al., 2019). Nanoparticles were further purified by dialysis for 24 h against 2 L of nanopure water (with changes of nanopure water after 4 and 16 h) using a Slide-A-Lyzer cassette with a 20,000 molecular weight cutoff.

Encapsulation of plasmid DNA within PLGA and ELP-PLGA particles and in vitro DNA release

For nanoparticles made with plasmid DNA, the vaccine backbone plasmid pVAC1 (InvivoGen) was transformed and produced in the *Escherichia coli* strain NEB 5-alpha (New England BioLabs). The plasmid was extracted using a plasmid Giga kit (Qiagen) and endotoxin was removed using an endotoxin removing column (Thermo Fisher). Polymer solution (100 mg dissolved in 2.5 mL of acetonitrile) was added dropwise to 7.5 mL filtered nanopure water containing vaccine backbone plasmid pVAC1 (InvivoGen). For stability studies, particles with 50 µg/mL of DNA were measured over 28 days. For *in vitro* release studies, particles with 24 µg/mL of DNA were characterized in 1x PBS (pH 7.4) over 8 days. Five milliliter volumes of P4-DNA nanoparticle solutions were shaken on an orbital shaker (100 rpm) at 4°C, 25°C, or 37°C. For each time point, 300 µL was removed, not returned to the reaction vial, and DNA concentration in the supernatant was measured. The loading and release of plasmid DNA in

nanoparticles was quantified by separation of PLGA nanoparticles from unencapsulated plasmid DNA by centrifugation, as described previously with minor modifications (Gomes dos Reis et al., 2019; Jo et al., 2020). Samples were centrifuged at 17,000 × g for 20 min and the supernatant was transferred to a new tube. The concentration of plasmid DNA remaining in the supernatant was measured using a Qubit dsDNA broad range assay kit and a Qubit fluorometer (Thermo Fisher Scientific). Encapsulation was expressed as the amount of plasmid initially loaded subtracted by the amount of plasmid in the supernatant, divided by the amount of plasmid initially loaded.

**ELP formulation and functionalization were created and performed at Los Alamos National Laboratory (CINT) by Mary Elizabeth Phipps*

Characterization of nanoparticles: Nanoparticle size, zeta potential, and concentration

PLGA nanoparticles were characterized for size and zeta potential using a Zetasizer Nano ZS (Malvern Instruments). Size standards were Nanosphere^{TM} polymer microspheres in water from Thermo Fisher Scientific. Vendor reported size values (NIST traceable) for 100 nm and 200 nm beads are as follows: mean diameter 100 nm ± 3 nm, size distribution 7.8 nm, standard deviation 7.8%, and 203 nm ± 5nm, size distribution 5.3 nm, and standard deviation 2.6%. Standards were diluted in 0.2 μm filtered nanopure water. Measurements were performed at 25°C (unless otherwise noted) in plastic disposable cuvettes. The standard operating procedure was as follows: number of measurements 3, number of runs per measurement 11, run duration 10 s each. Size is reported as diameter (nm) from 100% peak volume (volume corrected percentiles, not the z-average value nor measurements from intensity). Zeta potential transfer standards were from Malvern (DTS1235, -42 mV ± 4.2 mV) and used according to manufacturer's instructions.

Measurements were performed in Zetasizer folded capillary cells (DTS1070, Malvern). Zeta potential measurements cannot be made in concentrated salt solutions, such as is found in 1x PBS buffers (137 mM NaCl), as it decreases the ability to detect changes in particle charge. According to manufacturer's instructions (Malvern), zeta potential measurements of PLGA samples were taken in 0.1x PBS (13.7 mM NaCl, final concentration), not their standing 1x PBS buffer, so that the absolute zeta potential of particles could be accurately measured. Automatic measurement minimum runs were set at 10, maximum runs at 15, and number of measurements at 1-3. Particle concentration was measured using a NanoSight LM10-HSB (Malvern) equipped with a 405 nm laser. Particles were diluted 1:200 in nanopure water and measured for 60 s with manual gain and camera level settings. Captured videos were analyzed using the nanoparticle tracking analysis version 3.0 software. Between each reading, the sample chamber was cleaned with isopropanol followed by water.

Scanning electron microscopy (SEM) on PLGA nanoparticles

The particles were imaged by our collaborators at the Center of Integrated Nanotechnology (CINT) at Los Alamos National Laboratory (LANL) by Chris Sheehan as described below. Silicon wafers were cleaned by sonication in 100% ethanol for 8 minutes and then rinsed in nanopure water. Nanoparticles in water were dropcast (20 μl) onto individual wafers and were allowed to evaporate overnight at room temperature in a laminar flow hood. Samples were then imaged with a FEI Magellan 400 using an insertable concentric backscatter detector using 1 kV landing energy and 4 kV stage bias. Particle size and morphology was investigated using Image J (NIH) to calculate Feret's diameter and circularity.

Stability of PLGA nanoparticles

It is important to determine the stability of drug/vaccine delivery particles under conditions mimicking a physiological state, in order to ascertain the particles' usefulness to the biomedical field. In this study, nanoparticle stability under storage and physiological conditions was determined by keeping the particles in water at room temperature for up to 23 months, and in PBS (137 mM sodium chloride, pH 5.5 and 7.4) at 37°C (100 rpm on an orbital shaker) for up to 30 days. PLGA in PBS solutions were prepared by adding 100 μl of 10× concentrated PBS (Sigma Aldrich) to 900 μl of dialyzed nanoparticles. Measurements were made by DLS (Malvern Zetasizer).

Toxicity of PLGA nanoparticles

The toxicity of PLGA nanoparticles was completed by our collaborators at the Center of Integrated Nanotechnology (CINT) at Los Alamos National Laboratory (LANL) by Zachary R. Stromberg as described below.

Cell culture

The mouse fibroblast cell line L929 was obtained from ATCC (catalog number CCL-1). L929 cells are a standard cell line for cytotoxicity testing (Wallin and Arscott, 1998). Cells were cultured in Eagle's minimum essential medium containing 10% horse serum, maintained at 37°C in a 5% CO_2 incubator, and subcultivated at a ratio of 1:4, twice per week.

Cell viability assay

Cells were seeded at 5×10^3 cells per well in 96-well plates and incubated for 24 h before addition of nanoparticles. Supernatants were discarded and 100 μl of fresh media with

nanoparticles at various concentrations (10^4 to 10^9 particles/mL) were added. Cell culture media alone was used as a negative control and 6% methanol was used as a positive control for cytotoxicity. After 24 h, PrestoBlue solution (Invitrogen) was added at 10% final concentration to each well and plates were incubated at 37°C for 1 h. Fluorescence was measured using a SpectraMax Gemini EM plate reader with the excitation/emission wavelengths set at 560/590. An ANOVA followed by Tukey's method for multiple means comparison was used to compare experimental and control groups and a Student's t-test was used to compare PVA-P4 to P4 nanoparticles in cytotoxicity assays. Analyses were performed using GraphPad Prism version 8. *P* values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Characterization of nanoparticles made with different PLGA compositions

Knowing that different ratios of lactic and glycolic acid can change the PLGA particle size, degradation rate, half-life, and other factors, we looked into five PLGA compositions (P1 – P5) with different terminal functionality (Fig. 2). Each of the five PLGA nanoparticles were formulated by solvent evaporation method and characterized for size and zeta potential in water and PBS as a mimic for physiological conditions. As large shifts can occur in the ionic strength of cell and tissue fluid environments, it is important to understand nanoparticle behavior in both water and physiological solutions. Specifically, nanoparticle diffusion, aggregation, and degradation can be greatly affected by buffer conditions. In water, all PLGAs yielded approximately 150 nm particles, except for P1 which produced 234 nm particles (Table 1). Previous studies have shown that sodium chloride concentrations ≥10 mM can increase the size of PLGA nanoparticles (Huang and Zhang, 2018). In order to mimic physiologic conditions found in normal human serum, we used PBS, with a sodium chloride concentration of 137 mM (Kimura et al., 2014). When resuspended in PBS,

particle sizes increased to >1,000 nm for all PLGAs, except for P4 which remained stable in size (Table 1 and Fig. 3-4). As a secondary indicator of particle stability, the surface charge/zeta potential of nanoparticles in solution was also assessed. The general dogma has been that charged nanoparticles with a zeta potential less than -30 mV or greater than 30 mV have a high level of stability (Kumar and Dixit, 2017); however, stable nanoparticles with approximately neutral zeta potentials are found in the literature (Chigumira et al., 2015; Verma et al., 2017), as stability depends on the sum of the electrostatic repulsive forces and van der Waals attractive forces (Bhattacharjee, 2016). The zeta potential, in water, for the various PLGA (P1-P5) nanoparticles ranged from -47 to -62 mV (Table 1). However, after suspension in dilute PBS (0.1×), PLGAs with a carboxylic acid terminal group remained at -65 mV (P4) or decreased (-47 to -62 mV, P1). Whereas, PLGAs with an ester terminal group became more neutral (-26 mV, P2; 23 mV, P3; and -44 mV, P5), as is reasonable due to their surface functionalization. Given that the selection of PLGA formulations is often guided solely by degradation rate for controlled release, these results

indicate that PLGA selection must also consider size, zeta potential, and stability in physiological media in order to understand particle behavior *in vivo* and serve as an effective delivery system.

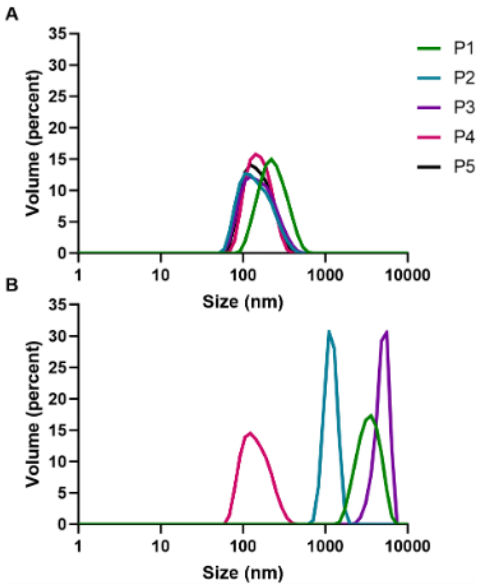


Figure 3. P4 forms the most stable PLGA nanoparticles (in PBS and water) of those PLGA examined. PLGA particle size was measured by dynamic light scattering in water (A) and PBS (B) for various PLGA (P1 – P5) particles formulated with different molecular weights, terminal groups, and lactic acid to glycolic acid ratios. The size of P5 (black) in PBS was greater than 10,000 nm and thus not shown in panel B. P1: 40-75 kDa; carboxylate terminated, 50:50 (lactic:glycolic repeat ratio); P2: 30-60 kDa, ester terminated, 50:50 (lactic:glycolic repeat ratio); P3: 40-75 kDa, ester terminated, 65:35 (lactic:glycolic repeat ratio); P4: 24-38 kDa, carboxylate terminated, 65:35 (lactic:glycolic repeat ratio); P5: 24-38 kDa, ester terminated, 50:50 (lactic:glycolic repeat ratio).

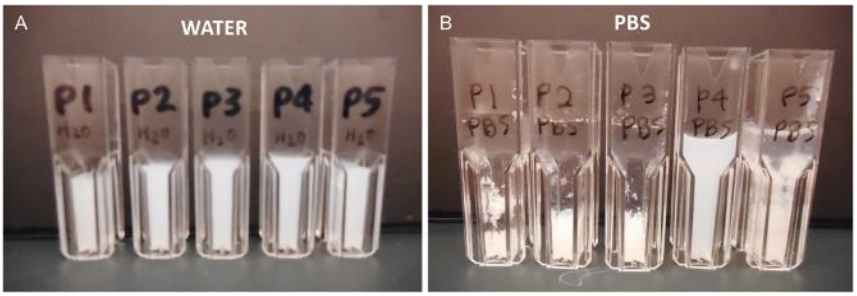


Figure 4. P4 forms the most stable particles (in PBS and water) of those PLGA examined, as visually demonstrated after 24 hours. PLGA nanoparticle (P1 – P5) solubility in water (A) and PBS (B) when formulated with different molecular weights, terminal groups, and lactic to glycolic acid ratios. P1: 40-75 kDa; carboxylate terminated, 50:50 (lactic:glycolic repeat ratio); P2: 30-60 kDa, ester terminated, 50:50 (lactic:glycolic repeat ratio); P3: 40-75 kDa, ester

terminated, 65:35 (lactic:glycolic repeat ratio); P4: 24-38 kDa, carboxylate terminated, 65:35 (lactic:glycolic repeat ratio); P5: 24-38 kDa, ester terminated, 50:50 (lactic:glycolic repeat ratio).

Table 1. P4 PLGA is the most stable for nanoparticle formulations. Characterization of particles made by solvent evaporation using PLGAs with different molecular weights, terminal groups, and lactic acid to glycolic acid ratios.

PLGA polymer	Size (nm) in water	PDI in water	Size (nm) in PBS	PDI in PBS	Zeta potential (mV) in water	Zeta potential (mV) in PBS
P1	233.6 ± 6.8	0.01 ± 0.03	4126.7 ± 773.6	0.42 ± 0.19	-47.4 ± 6.5	-61.5 ± 9.0
P2	150.3 ± 4.3	0.16 ± 0.02	1972.8 ± 2123.3	0.66 ± 0.37	-52.6 ± 7.4	-25.8 ± 25.2
P3	158.6 ± 6.7	0.15 ± 0.01	4916.3 ± 117.0	0.07 ± 0.03	-48.9 ± 7.9	-22.5 ± 11.1
P4	154.5 ± 5.7	0.08 ± 0.02	147.7 ± 2.9	0.11 ± 0.02	-62.4 ± 8.0	-65.7 ± 12.1
P5	152.5 ± 4.5	0.07 ± 0.03	29333.3 ± 16502.5	0.86 ± 0.12	-53.2 ± 7.7	-44.1 ± 6.9

Size and PDI (polydispersity index) are reported as a mean of 3 measurements from one representative sample ± SD and zeta potential as ± zeta deviation. For nanoparticles suspended in PBS, size measurements were made in 1× PBS and zeta measurements were made in 0.1× PBS (13.7 mM NaCl). The zeta potential measurements were made in 0.1× PBS, by diluting samples

stored in 1X PBS with water immediately prior to measurement, in order to better understand absolute zeta potential of particles per manufacturer's instructions (Malvern).

As a result of size and surface charge stability, P4 nanoparticles were used in all subsequent experiments. It is hypothesized that the lower molecular weight (24-38 kDa) and high lactic:glycolic acid ratio (65:35) of the P4 PLGA polymer results in increased intermolecular interactions, especially within the lactic acid core, while the carboxyl terminal groups remain strongly electronegative (Table 1) in both solvents, preventing disruption of particle packing in ionic solutions (Slimane et al., 2020). Furthermore, the carboxylic acid terminal group of P4 provides for facile surface functionalization using several straightforward amide coupling reactions. For example, therapeutic and immunostimulatory molecules containing amine groups can be easily conjugated to the carboxylic acid groups on the surface of P4 nanoparticles for vaccine delivery systems (Sah et al., 2013). This behavior allows for additional tunability of P4 nanoparticles to control stability, degradation rate, and cellular interaction.

Characterization of PVA-P4 nanoparticles

Stabilizers such as PVA are nearly always used in particle formation and long-term stability (Swider et al., 2018). Unfortunately, incorporation of PVA into PLGA formulations typically requires probe sonication, which can degrade any biological material (e.g. DNA) included in the formulation (Grabski, 2009; Wang et al., 2014). To avoid these issues, PVA-P4 nanoparticles were originally made by the same solvent evaporation methods as we delineate above for preparation of PLGA particles, without sonication; unfortunately, those methods were not further pursued due to large variability in nanoparticle size/very high polydispersity (data not shown). Instead, P4

nanoparticles were formulated with PVA using the gold standard single-emulsion method, with sonication, and size and zeta potential were measured at different formulation steps. Nanoparticles made with 50 mg of P4 and 4% PVA had a size of 302 nm post-sonication (Fig. 14) and had a neutral zeta potential (Table 2 and Fig. 05). After overnight solvent evaporation, the particle size decreased to 141 nm and zeta potential remained neutral (-1.7 mV). Variation in PVA concentration (1% vs 4%) altered the size, with 1% PVA-P4 producing 250 nm particles, while 4% PVA-P4 nanoparticles were ~150 nm. The difference in size between these formulations is likely due to a lower interfacial energy with PVA on the surface of nanoparticles, as well as increased viscosity during formation which allow for the formation of smaller particles (Slimane et al., 2020).

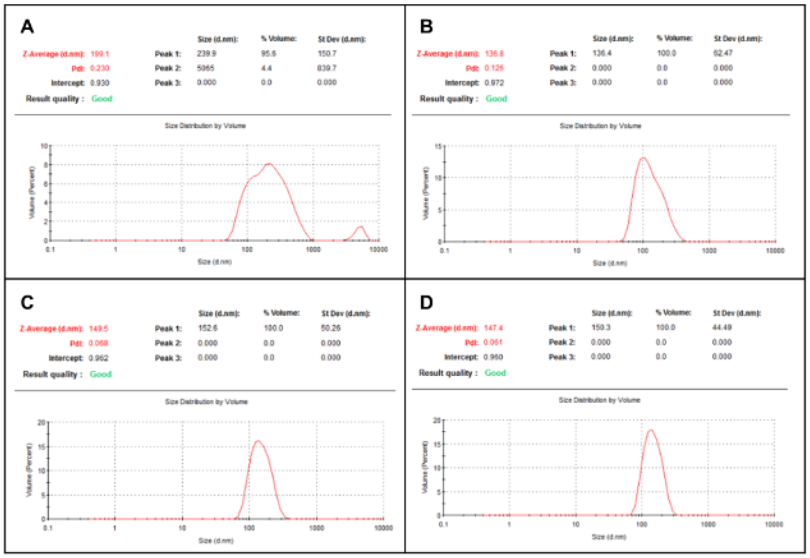


Figure 5. PVA-P4 particle size distribution evolves throughout the formulation process post-sonication (A), post-solvent evaporation (B), post-centrifugation (C), and post-dialysis (D). Representative light-scattering size measurements of PVA-P4 nanoparticles are shown here (1 of 3 measurements, averages shown in Table 2). While significant polydispersity is observed in PVA-P4 nanoparticles following sonication and solvent evaporation, centrifugation and dialysis results in more monodisperse particles.

Table 2. PVA-P4 particle size and surface charge distribution evolves throughout the formulation process. Physical characterization of PVA-P4 nanoparticles at multiple steps during the single-emulsion method.

Formulation step	Size (nm)	PDI	Zeta potential (mV)
Post-sonication	301.9 ± 69.9	0.24 ± 0.02	-0.8 ± 4.8
Post-solvent evaporation	141.0 ± 10.4	0.12 ± 0.003	-1.7 ± 3.7
Post-centrifugation	154.4 ± 6.3	0.06 ± 0.01	-11.3 ± 4.8
Post-dialysis	154.3 ± 3.6	0.06 ± 0.01	-11.7 ± 4.8

Size and PDI (polydispersity index) are reported as a mean of 3 measurements from one representative sample ± SD and zeta potential as ± zeta deviation.

As part of the single-emulsion method, centrifugation following solvent evaporation is the most common method to remove residual cytotoxic PVA, while dialysis is sometimes added after centrifugation for further purification (Birnbaum et al., 2000; Jonderian and Maalouf, 2016). The slight increase in P4 particle size from 141 to 154 nm along with a decrease in polydispersity following centrifugation and dialysis (Fig. 14) could correspond to loss of surface bound PVA and reorganization of PLGA. However, the zeta potential of PVA-P4 nanoparticles prior to and following centrifugation and dialysis were -11.3 and -11.7 mV, respectively. Relative to P4 PLGA particles alone (-62.4 mV) the lack of equivalent negative charge in the PVA-P4 particles implies centrifugation and dialysis are insufficient for complete removal of PVA (Fig. 16). Such

inconsistencies led to our investigation of whether PVA was necessary to generate long-term, stable P4 nanoparticles with consistent and reproducible characteristics.

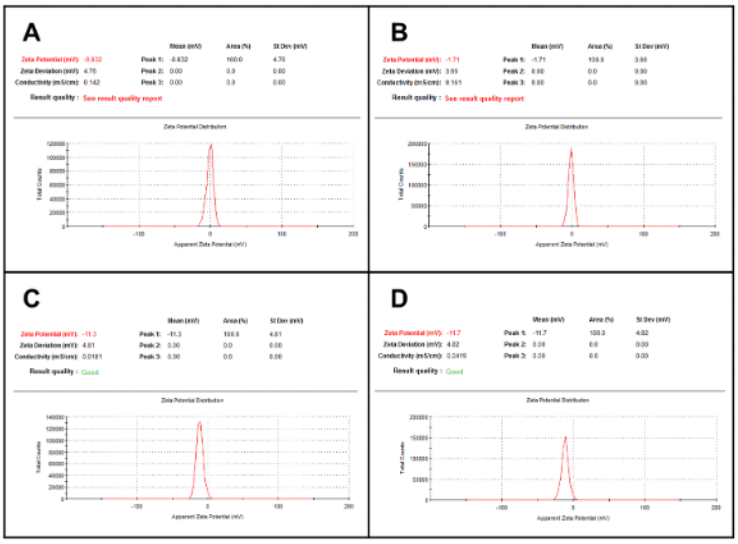


Figure 6. PVA-P4 particles surface charge/zeta potential evolves throughout the formulation process post-sonication (A), post-solvent evaporation (B), post-centrifugation (C), and post-dialysis (D). Zeta potential was significantly more neutral than P4 nanoparticles alone (-60 mV), (-0.8 mV) post-sonication (A), (-1.7 mV) post-solvent evaporation (B), and became slightly more electronegative, (-11.3 mV) post-centrifugation (C), and (-11.7 mV) post-dialysis (D). These results imply that PVA removal is variable and that the surface characteristics of PVA stabilized PLGA particles may vary depending upon processing.

P4 nanoparticles are as stable as PVA-P4 nanoparticles

Stabilizer-free PLGA nanoparticles are rarely used in the literature because they can be challenging to prepare and store during transport (Cheng et al., 2008; Grabowski et al., 2015; Gyulai and Kiss, 2017; Tomoda et al., 2014). Therefore, a simple method that creates stabilizer-free nanoparticles with similar long-term stability comparable to the gold standard (PVA-stabilized), is critical to the development of PLGA delivery systems. Previous work on stabilizer-free PLGA particle formations has shown some improvement in cellular viability over the PVA gold standard; however, long-term stability under physiological conditions has yet to be extensively investigated as an alternative to PVA-stabilized methods, as we have completed here.

[Grabowski, et al., Int. J Pharm. 2015; Stolnik, et al., Colloids Surf. 1995; Gyulai, Kiss, J Colloid. Inter. 2017; Cheng, et al. Biomaterials 2008; Albert, et al. Langmiur 2018.] When visualized using SEM, (Fig. 7) both P4 (115.49 ±72.77 nm) and PVA-P4 (146.54 ± 96.00 nm) nanoparticles demonstrated a spherical morphology (circularity ≥ 0.95) with sizes consistent with solution DLS measurements of 126 nm and 154 nm, respectively. Stability of P4 and PVA-P4 nanoparticles following formation and storage was assessed using size and zeta potential measurements taken for at least 21 days at room temperature in water and PBS storage conditions (Fig. 8). During these experiments, stabilizer-free P4 remained as stable in both water and PBS as PVA-P4, with no significant change in particle size (Fig. 8A). The size of P4 nanoparticles remained at approximately 150 nm for at least 30 days in water and PBS, highlighting P4 nanoparticle stability in solution without the need for lyophilization or refrigeration. In addition to size stability, surface charge of P4 and PVA-P4 nanoparticles followed previous experiments, with -63 mV for P4 and -15 mV for PVA-P4 in water (Fig 8B). Over at least 21 days, PVA-P4 nanoparticles remained either neutral or around -15 mV, while P4 nanoparticles remained significantly more electronegative (-50 to -70 mV). Overall, these results show that for size and zeta potential, P4 nanoparticles are as stable as PVA-P4 nanoparticles over time. In addition to stability, generating highly negative charged nanoparticles may be advantageous for several applications. For example, transdermal drug delivery of negatively charged PLGA nanoparticles (made with 10 kDa, 75:25 PLGA) led to 53 to 77 times higher electrophoretic mobility compared to PVA-PLGA

nanoparticles resulting in greater tissue distribution for drug delivery by iontophoresis (Tomoda et al., 2014).

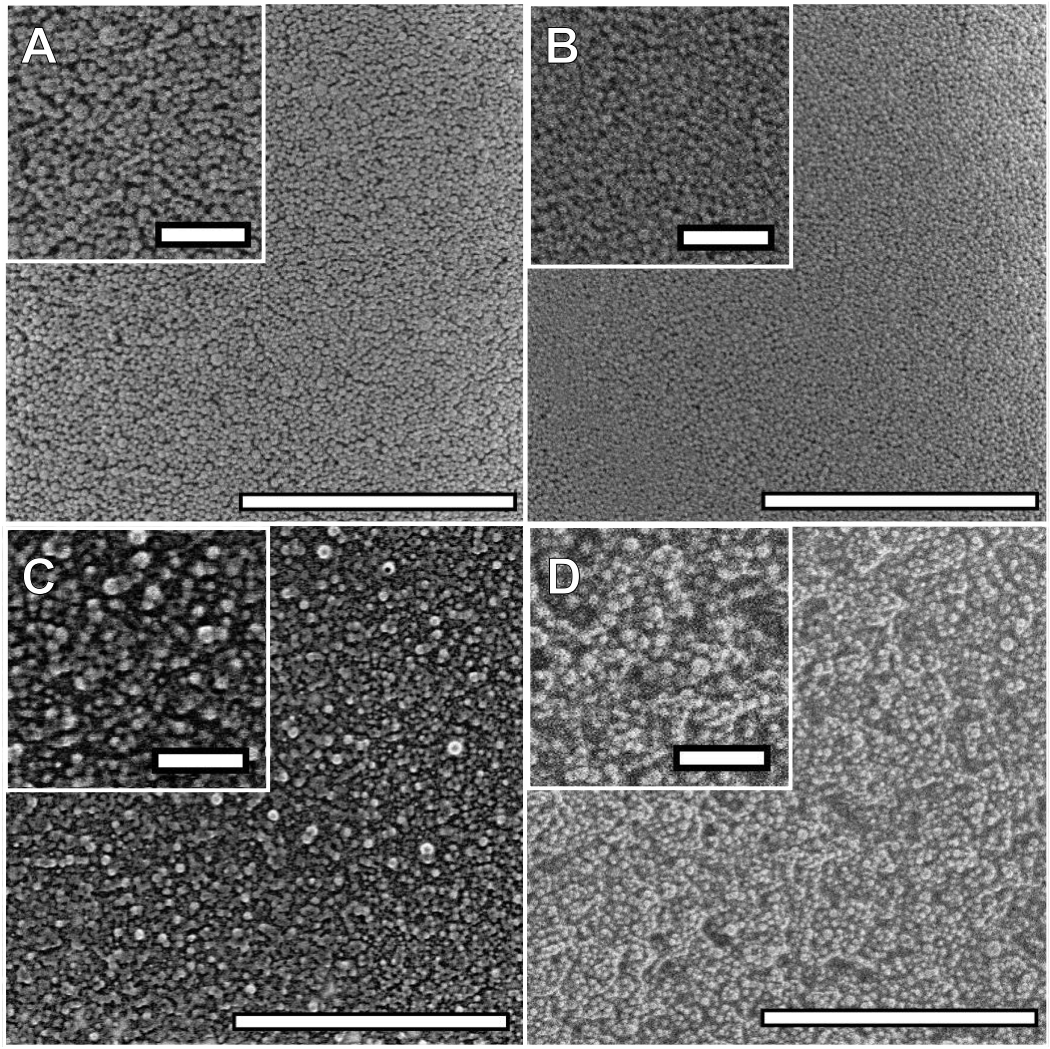


Figure 7. P4 nanoparticles exhibit a spherical morphology when visualized using scanning electron microscopy. PLGA nanoparticles fabricated with P4 (A), P4-DNA (B), PVA-P4 (C), and ELP-P4 (D). Particle size, as determined by Image J analysis, was consistent with solution based measurements by dynamic light scattering (e.g. P4 by DLS = 126 nm, P4 by SEM = 115.49 72.77 nm, PVA-P4 by DLS = 154 nm, PVA-P4 by SEM = 146.54 96.00 nm). Scale bars are 5 m for the main images and 1 m for the insets. P4 and ELP- P4 particle aggregation was driven by the sample drying conditions, and not observed in solution when characterized by DLS. This feature was especially noted in panel D, likely due to the propensity of ELP nanomaterials to coacervate upon an increase in concentration.

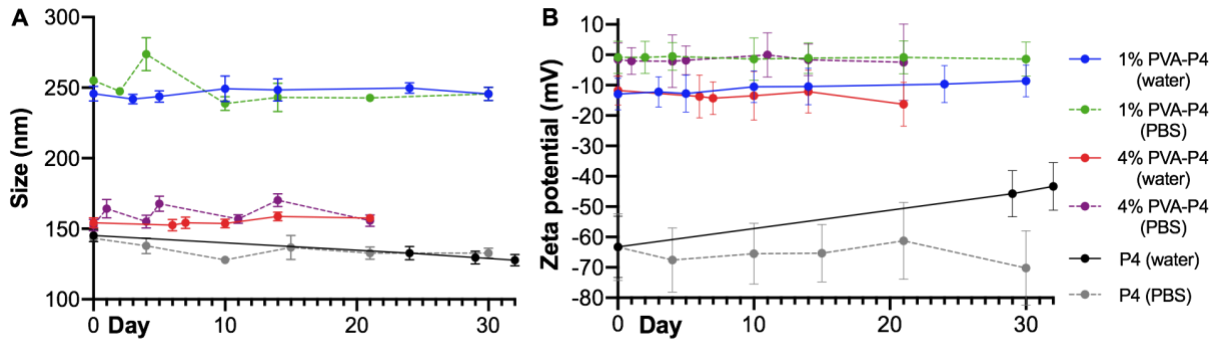


Figure 8. P4 nanoparticles are as stable as PVA-P4 over 30 days. P4 nanoparticle size (A) and zeta potential (B), measured by dynamic light scattering, with and without the inclusion of PVA. P4 was consistently stable over a 30-day period and maintained a highly negative zeta potential whereas PVA-P4 particles were stable, but significantly more neutral. Nanoparticles were prepared by single-emulsion (PVA-P4) or solvent evaporation (P4). Samples in PBS were diluted 1 in 10 in water to measure zeta potential. Data are reported as a mean of three size measurements of one representative sample ± SD and zeta potential ± zeta deviation.

P4 nanoparticles provide carriers for DNA vaccines

In order to create vaccines using nanoparticle delivery systems, protein antigens, plasmid DNA, or mRNA is typically encapsulated for transport to target cells with release over time. As vaccine carriers, particles with a diameter >500 nm are commonly taken up by dendritic cells or macrophages via phagocytosis for transport to lymph nodes, mid-sized particles (200-500 nm) are more selectively taken up by dendritic cells (Silva et al., 2015), and smaller sized particles (20-200 nm) travel directly to lymphatic vessels and into the draining lymph nodes (Shima et al., 2013). While all can activate the immune system, special attention must be made to controlling particle size in order to induce an increased immune response in the desired cell population and limit the need for multiple doses to build up or maintain immunity (Manolova et al., 2008; Reddy et al., 2007; Zhang et al., 2017). By controlling the synthesis parameters, nanoparticle properties such as size, charge, and surface potential can be tuned for efficient uptake by the target cells (Mittal et al., 2007). Previously, ionic strength, solvent dispersion, polymer concentration, and temperature

have been explored for tuning the size of PLGA nanoparticles (Huang and Zhang, 2018). Variations in PLGA:water stoichiometry (1.67 to 26.67 mg/mL P4) were used to alter particle size for the creation of a tunable stabilizer-free PLGA delivery system (Fig. 9), as has been shown previously for controlling microparticle size (Mensah et al., 2019). Additionally, plasmid DNA was added through the aqueous phase to determine whether DNA could be effectively incorporated, and if it would influence particle packing or alter size and surface characteristics.

Increasing the concentration of P4 polymer from 1.67 to 13.33 mg/mL resulted in an increase in nanoparticle size. The average sizes of three measurements were 93 nm for 1.67 mg/mL, 153 nm for 3.33 mg/mL, 160 nm for 6.67 mg/mL, and 222 nm for 13.33 mg/mL (-75 mV zeta potential, Table 3 and Fig. 9-10). At 20 mg/mL (220 nm), the size tended to plateau (210 nm, 26.67 mg/mL). In addition, we observed that nanoparticles formulated with P4 concentrations < 6.67 mg/mL were very highly monodisperse, as indicated by polydispersity index (PDI) values below 0.1 (Table 3). However, nanoparticles formulated with P4 concentrations ≥ 6.67 mg/mL were relatively more polydisperse with PDI values ranging from 0.1 to 0.31, which may lead to some variation in size between batches. While the addition of plasmid DNA during formulation (P4-DNA) did not have a major impact on size of P4 nanoparticles, a greater negative charge was observed for most P4-DNA formulations as compared to all stoichiometrically similar P4 nanoparticles (Table 3 and Fig. 10). Increasing the concentration of P4 polymer from 1.67 to 20 mg/mL resulted in a decrease in the zeta potential. At 26.67 mg/mL of P4 the zeta potential became more positive with a value of -66 mV (Table 3). Given the negative charge of PLGA and DNA, only 10 to 15% of plasmid DNA (5-7.5 µg/mL) was encapsulated for these P4 nanoparticles (Table 3), a concentration which has been shown suitable for vaccination (Jo et al., 2020).

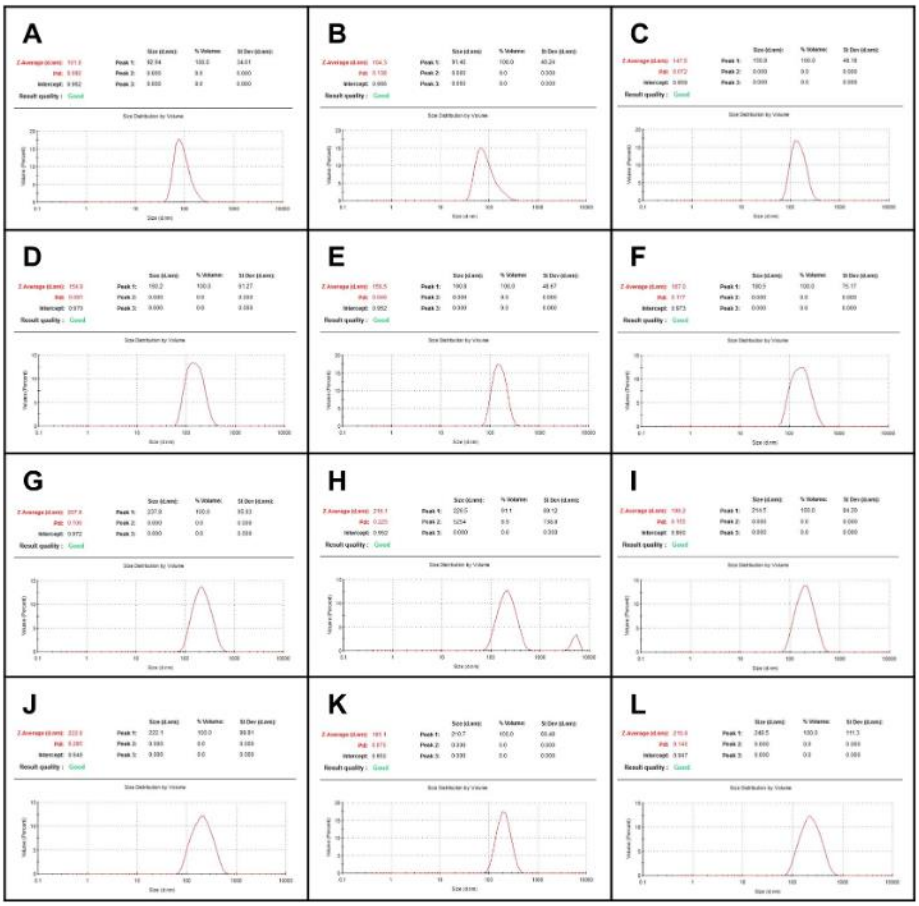


Figure 9. Particle size can be controlled through stoichiometry of P4 to water and addition of DNA in formulation process. Representative light scattering data presented within Table 3 (main manuscript) for size of P4 nanoparticles formulated with various P4 and DNA concentrations. Representative sizes of nanoparticles formulated with: 1.67 mg/mL P4 (A), 1.67 mg/mL P4 plus 50 μg/mL of DNA (B), 3.33 mg/mL P4 (C), 3.33 mg/mL P4 plus 50 μg/mL of DNA (D), 6.67 mg/mL P4 (E), 6.67 mg/mL P4 plus 50 μg/mL of DNA (F), 13.33 mg/mL P4 (G), 13.33 mg/mL P4 plus 50 μg/mL of DNA (H), 20 mg/mL P4 (I), 20 mg/mL P4 plus 50 μg/mL of DNA (J), 26.67 mg/mL P4 (K), and 26.67 mg/mL P4 plus 50 μg/mL (L). Three measurements were performed for each sample, above is the size data for a representative measurement within that average.

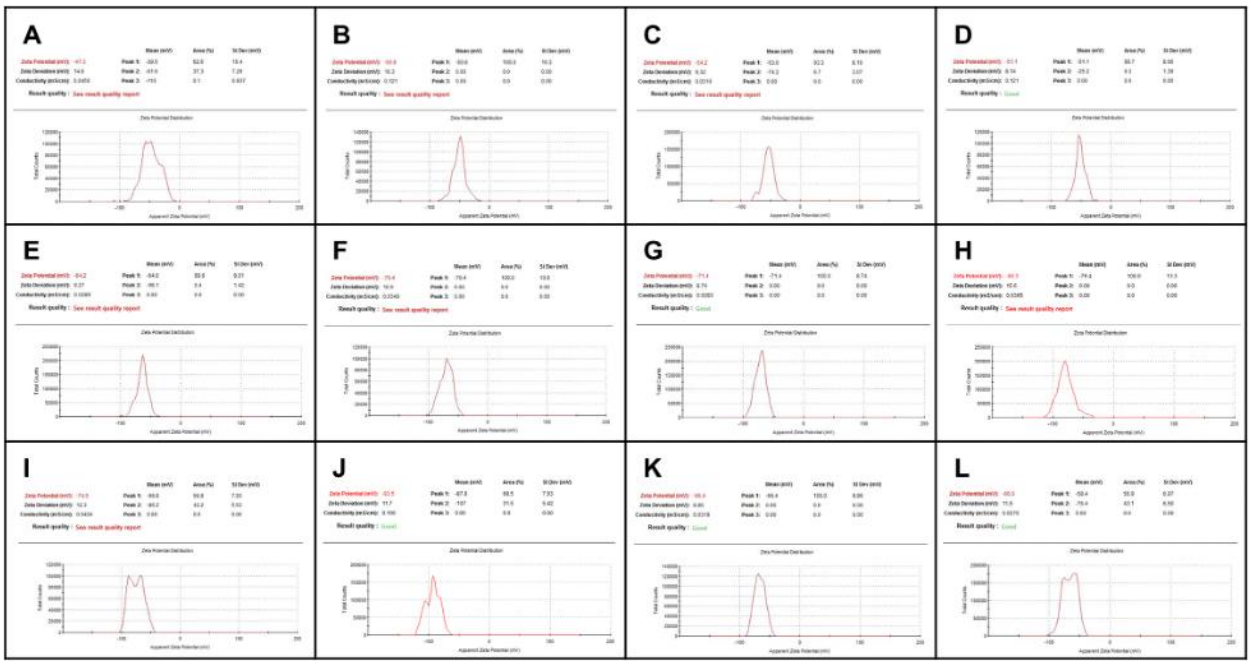


Figure 10. Surface charge is largely invariant upon change of P4 particle size, yet addition of DNA to the P4 formulation trends toward a more negative surface charge. Representative zeta potential of PLGA nanoparticles formulated with various polymer and DNA concentrations (Table 3, main manuscript). Representative sizes of nanoparticles formulated with: 1.67 mg/mL P4 (A), 1.67 mg/mL P4 plus 50 µg/mL of DNA (B), 3.33 mg/mL P4 (C), 3.33 mg/mL P4 plus 50 µg/mL of DNA (D), 6.67 mg/mL P4 (E), 6.67 mg/mL P4 plus 50 µg/mL of DNA (F), 13.33 mg/mL P4 (G), 13.33 mg/mL P4 plus 50 µg/mL of DNA (H), 20 mg/mL P4 (I), 20 mg/mL P4 plus 50 µg/mL of DNA (J), 26.67 mg/mL P4 (K), and 26.67 mg/mL P4 plus 50 µg/mL (L).

Table 3. Impact of P4 and DNA concentration on nanoparticle size and zeta potential.

P4 conc. (mg/mL)	DNA conc. (µg/mL)	DNA encapsulation (%)	Size (nm)	PDI	Zeta potential (mV)
1.67	0	-	93.3 ± 1.4	0.09 ± 0.01	-47.3 ± 14.8
1.67	50	15.6	92.2 ± 1.9	0.14 ± 0.02	-50.8 ± 10.3
3.33	0	-	153.0 ± 3.8	0.06 ± 0.01	-54.2 ± 9.3
3.33	50	10.8	162.9 ± 8.5	0.09 ± 0.02	-51.1 ± 8.1
6.67	0	-	160.0 ± 1.5	0.08 ± 0.04	-64.2 ± 9.3

6.67	50	16.4	182.2 ± 8.6	0.10 ± 0.02	-70.4 ± 10.9
13.33	0	-	222.0 ± 13.9	0.10 ± 0.02	-71.4 ± 8.7
13.33	50	16.4	232.1 ± 15.4	0.23 ± 0.01	-80.2 ± 15.6
20	0	-	219.7 ± 17.6	0.18 ± 0.03	-74.9 ± 12.3
20	50	16.0	247.7 ± 50.6	0.31 ± 0.03	-93.5 ± 11.7
26.67	0	-	210.2 ± 2.0	0.10 ± 0.03	-66.4 ± 8.9
26.67	50	13.2	255.6 ± 17.3	0.17 ± 0.03	-66.0 ± 11.6

Samples were prepared by solvent evaporation and measured in water. Size values are presented as the mean of one representative sample ± SD of three measurements and zeta potential ± zeta deviation. PDI, polydispersity index; -, not applicable.

P4-DNA nanoparticles are as stable as P4 nanoparticles

Having demonstrated that P4 nanoparticles are as stable as PVA-P4 nanoparticles at room temperature (Fig. 8), we then sought to assess P4 and P4-DNA nanoparticle stability at 37°C (Fig. 12). Particles of 300 nm (13.33 mg/mL P4) were made with and without a vaccine plasmid (50 µg/mL, 15 to 22% encapsulated) and monitored for stability over 28 days (Fig. 12). As a mimic for interstitial fluid, nanoparticles were suspended in PBS at pH 7.4 and 37°C. The size of P4 and P4-DNA nanoparticles remained at approximately 300 nm for 14 days (Fig. 12A and Fig. 11). On day 21, P4 nanoparticles increased in size to >1,000 nm, while P4-DNA particles had a size of 346 nm. By day 28, the size of P4 particles was unable to be measured by DLS, indicating particle degradation/flocculation. The size of P4-DNA nanoparticles was also unstable and had increased to 800 nm on day 28. Under the same conditions, zeta potential followed a similar trajectory as size and remained stable between -62 to -82 mV for 14 days. On day 21, P4 nanoparticles had become more positive (-22 mV), while the P4-DNA nanoparticle solution had a zeta potential of -

74 mV. By day 28, P4 nanoparticles had reached a neutral zeta potential, while P4-DNA nanoparticle solutions were -58 mV (Fig. 12C). In addition, P4-DNA nanoparticles suspended in PBS at pH 7.4 were assessed at 37°C, room temperature (25°C), and 4°C for *in vitro* plasmid release (Fig. 13). Similar release profiles were observed for P4-DNA nanoparticles at 25°C and 37°C, while release at 4°C remained at ~30 to 40% over 8 days. Although we used the standard centrifugation method described previously to quantify DNA release (Gomes dos Reis et al., 2019; Jo et al., 2020), the results should be interpreted with caution because centrifugation may affect particle stability and subsequently release plasmid DNA from these particles. No size or zeta potential changes were observed over 8 days (Fig. 13).

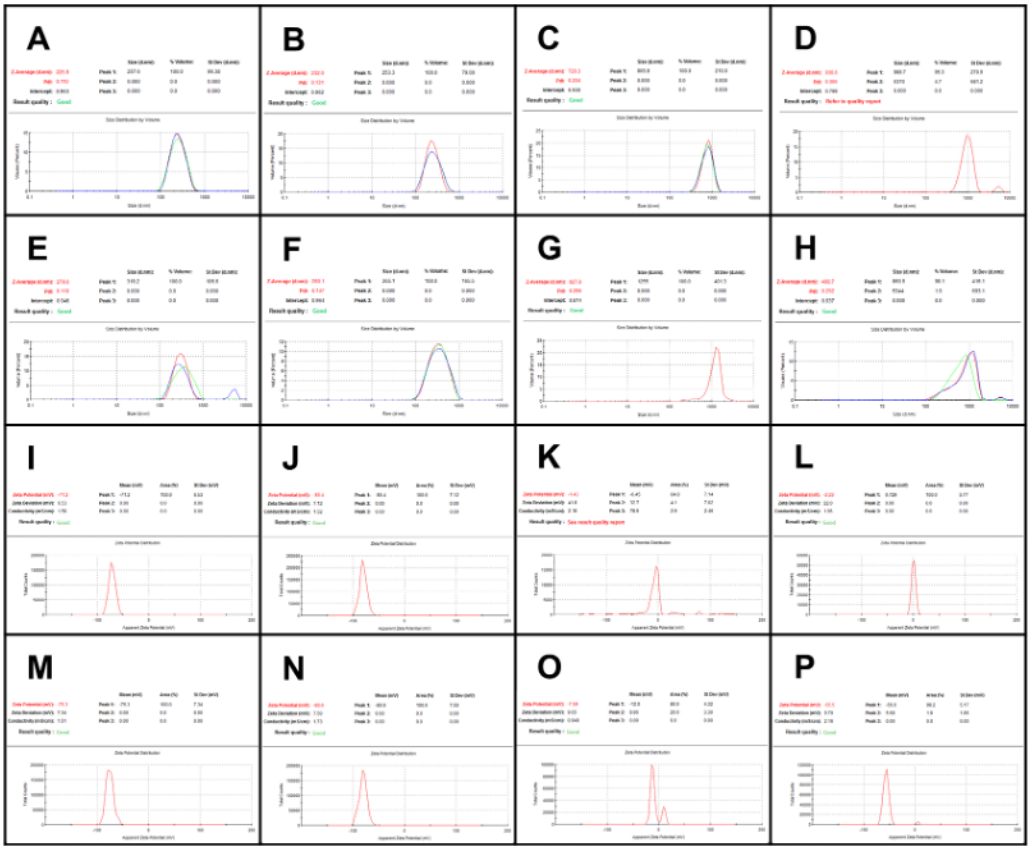


Figure 11. Nanoparticles made with plasmid DNA are as stable as P4 only nanoparticles. Three independent samples were measured over 28 days and representative data are shown for each

measurement at day zero and the last day where samples were still measurable before degradation (Data represented in Fig 3, main manuscript). Size plots (100% peak volume) are shown for P4 at pH 5.5 on day 0 (A), P4 at pH 7.4 on day 0 (B), P4 at pH 5.5 on day 14 (C), P4 at pH 7.4 on day 21 (D), P4-DNA at pH 5.5 on day 0 (E), P4-DNA at pH 7.4 on day 0 (F), P4-DNA at pH 5.5 on day 21 (G), and P4-DNA at pH 7.4 on day 28 (H). Plots for zeta potential measurements are shown for P4 at pH 5.5 on day 0 (I), P4 at pH 7.4 on day 0 (J), P4 at pH 5.5 on day 28 (K), P4 at pH 7.4 on day 28 (L), P4-DNA at pH 5.5 on day 0 (M), P4-DNA at pH 7.4 on day 0 (N), P4-DNA at pH 5.5 on day 28 (O), and P4-DNA at pH 7.4 on day 28 (P).

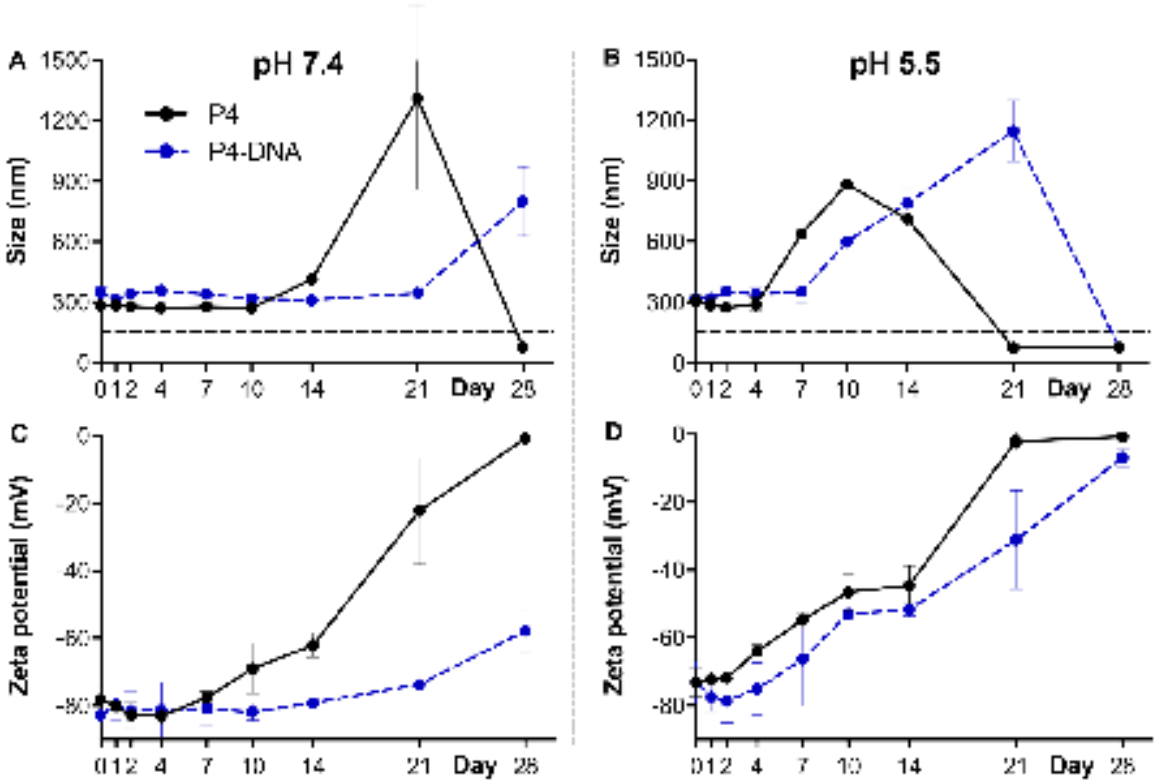


Figure 12. Nanoparticles made with plasmid DNA are as stable as P4 only nanoparticles. P4 nanoparticle size (A, B) and zeta potential (C, D) at human body temperature (37 C) with and without the inclusion of plasmid DNA, were measured as a function of pH. Nanoparticles were

prepared by the solvent evaporation method and incubated on an orbital shaker (100 rpm) at C in PBS at a pH of 7.4 (A, C) or 5.5 (B, D). Data are presented as a mean of three independent samples SD. Samples that had an undetectable size were assigned a value below the horizontal dashed line.

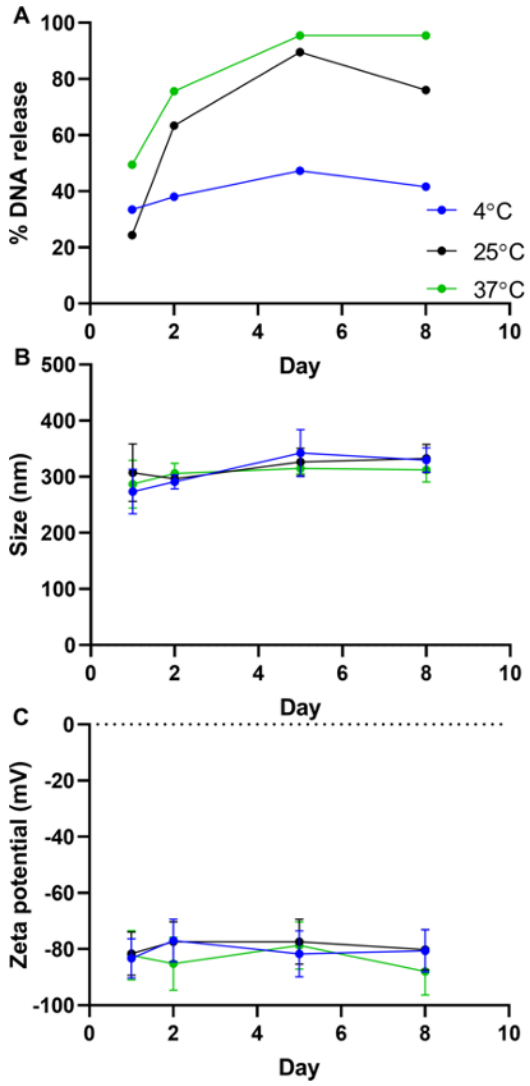
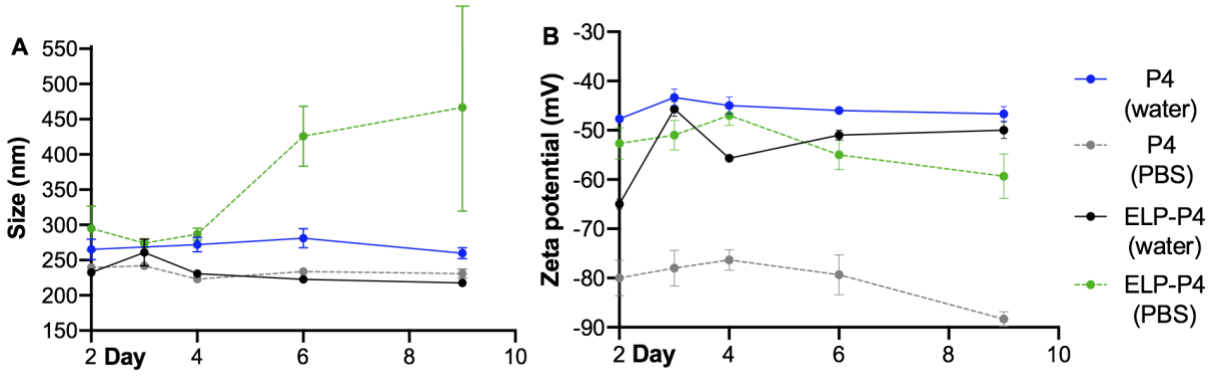


Figure 13. Profiles of plasmid DNA released from P4-DNA nanoparticles. Nanoparticles were formulated with 24 µg/ml of plasmid DNA. DNA concentration was measured over 8 days from 300 µL aliquots (5 mL initial total volume for each temperature condition) of P4-DNA

nanoparticle solutions suspended in 1× PBS (pH 7.4) shaken on an orbital shaker (100 rpm) at 4°C (blue), 25°C (black), or 37°C (green) (A). Nanoparticle size measurements (B) were made in 1× PBS and zeta potential measurements (C) were made in 0.1× PBS by dynamic light scattering. One sample was tested per condition. DNA release was reported from one measurement per timepoint, size as a mean of three measurements ± SD, and zeta potential ± zeta deviation.

After migration through interstitial fluid, nanoparticles enter cells and are transported into endosomes (Xia et al., 2016). In order to mimic the acidic environment of endosomes (Hu et al., 2015), experiments were run in PBS solutions at pH 5.5 and 37˚C. Both P4 and P4-DNA nanoparticle size remained around 300 nm for 4 days (Fig. 4B and Fig. S9). By day 7, P4 nanoparticle size increased to 638 nm, while P4-DNA nanoparticles were 352 nm. On day 14, both P4 and P4-DNA nanoparticles had sizes around 700 nm, and by day 21, P4 nanoparticle size was not measurable by DLS, indicating particle degradation and erosion. In contrast, P4-DNA particles only reached a size >1,000 nm on day 21. Under the same conditions, zeta potential of both P4 and P4-DNA nanoparticles remained stable for two days and then increased almost linearly over time until a neutral charge was reached (Fig. 4D). Slower degradation of P4 nanoparticles at pH 7.4 is an important characteristic for use as delivery systems as it enables longer periods for tissue migration and cellular uptake before degradation and release of the DNA cargo can occur within cellular vesicles at a lower pH (Hu et al., 2015). Overall, these results demonstrate that P4-DNA nanoparticles are as stable as P4 only nanoparticles while tuned to allow for cargo release within cellular vesicles.

In order to further assess the effects of P4 nanoparticle size on stability under physiological conditions, nanoparticles of 200, 299, and 310 nm were assessed in PBS at 37°C in acidic and neutral environments (Fig. S11). In pH 7.4, the size and zeta potential of all P4 nanoparticles remained stable for 14 days (Fig. S11A). On day 21, particle size and zeta potential increased to 323 nm and -65.1 mV (6.67 mg/mL), 1,311 nm and -22 mV (13.33 mg/mL), and 766 nm and -45 mV (20 mg/mL), respectively. By day 28, the nanoparticles made with 6.67 mg/mL of P4 had increased in size to 533 nm and surface charge of -49 mV, while the other particles sizes were not measurable by DLS and had reached neutral surface charge. These results suggest that the nanoparticles that were initially larger (299 and 310 nm) degraded faster than smaller (200 nm) nanoparticles. At pH 5.5, the size and zeta potential profiles for all three initial particles sizes (200, 299, and 310 nm) of P4 particles followed a similar trajectory over 28 days (Fig. 15 B & D), with degradation beginning to occur between 1 to 2 weeks. These results indicate nanoparticle size can be used to tune degradation (Amann et al., 2010) within a 2 to 4 week period that matches the often used prime-boost schedule for sustained delivery of vaccine molecules (Bhardwaj et al., 2020).



**ELP formulation and functionalization were created and performed at Los Alamos National Laboratory (CINT) by Mary Elizabeth Phipps*

Figure 14. ELP-P4 nanoparticles are as stable as P4 nanoparticles in water but not PBS. P4 nanoparticle size stability (A) and zeta potential (B) with and without elastin conjugation (ELP-

P4), measured as a function of solvent (water and PBS) over 9 days. P4 nanoparticles in PBS exhibited the greatest stability in size and charge over 9 days. ELP-P4 maintained the same stability in water, as P4 only. However, ELP-P4 particles did begin to degrade in PBS at an earlier time point. Data are reported as a mean of three size measurements of one representative sample SD and zeta potential zeta deviation.

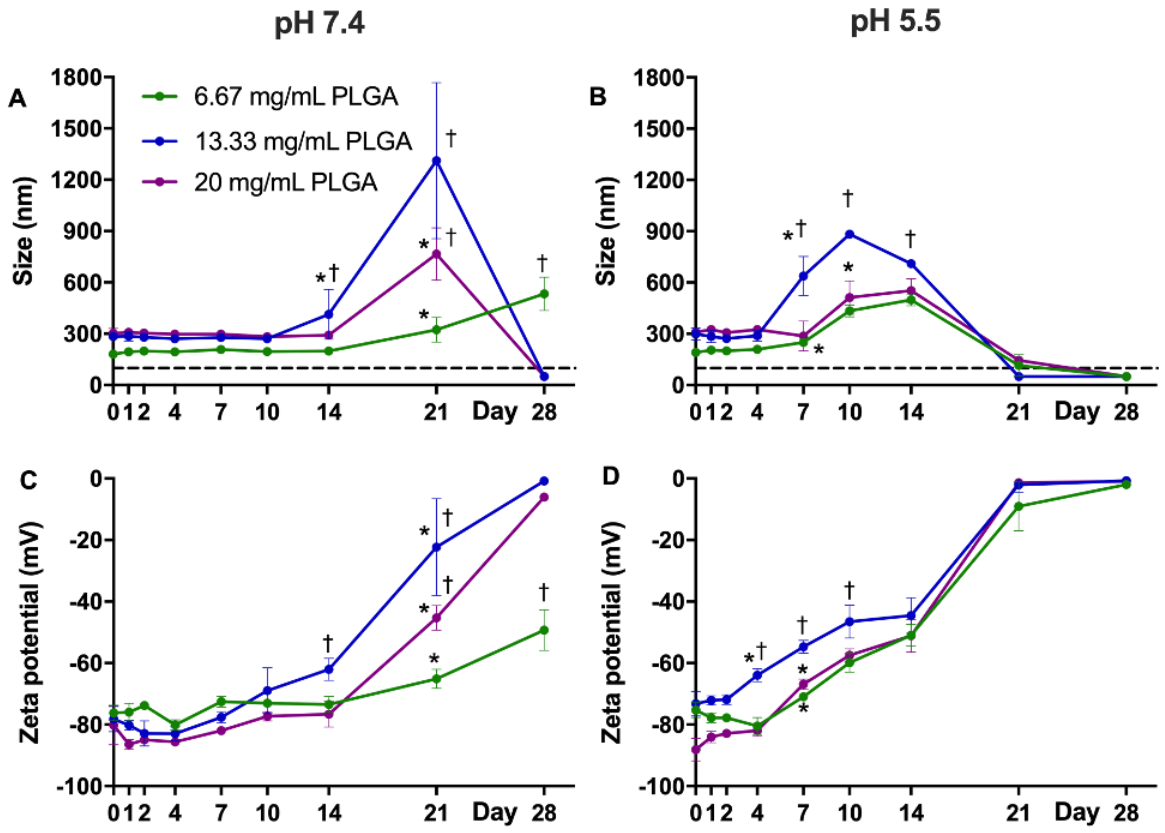


Figure 15. Smaller P4 nanoparticles are more stable than larger P4 nanoparticles after 28 days at 37°C in pH 7.4. The size (A, B) and zeta potential (C, D) of P4 nanoparticles made with 6.67 mg/mL, 13.33 mg/mL, and 20 mg/mL were measured at human body temperature (37˚C), as a function of pH. Nanoparticles were prepared by the solvent-evaporation method and incubated on an orbital shaker (100 rpm) at 37°C in PBS adjusted to pH of (A,C) 7.4 or (B, D) 5.5. Samples that had an undetectable size were assigned a value below the horizontal dashed line. *

Significant increase from previous timepoint, $p<0.05$, $n=3$; † significant difference between groups, $p<0.05$, $n=3$.

P4 and ELP-P4 nanoparticles are non-cytotoxic

The following ELP formulation and functionalization results were performed at Los Alamos National Laboratory (CINT) by Zachary Stromberg. In order to assess nanoparticle cytotoxicity, P4 nanoparticles alone, or modified with PVA, DNA, elastin, or with elastin and DNA were evaluated through incubation of L929 fibroblasts with nanoparticles ranging in concentration from 10^4 to 10^9 particles/mL. Importantly, PVA-P4 nanoparticles were cytotoxic, while all other nanoparticle formulations were non-toxic (Fig. 16). Based on the neutral zeta potential of P4-PVA nanoparticles following centrifugation and dialysis (Fig. 3), PVA is ineffectively removed, accounting for cellular cytotoxicity. For all concentrations tested, cells exposed to PVA-P4 nanoparticles had significantly ($P < 0.05$) reduced viability compared to P4 treated cells with a marked increase in toxicity at higher concentrations (Fig. 16B). In contrast, P4 and ELP-P4 nanoparticles were nontoxic, and the 10^4 particle/mL concentration of ELP-P4 nanoparticles exhibited significantly higher ($P = 0.04$) cellular viability compared to all other treatments. These results indicate that ELP conjugated P4 particles are nontoxic and could be beneficial for enhancing the delivery of therapeutic cargos (Cho et al., 2016).

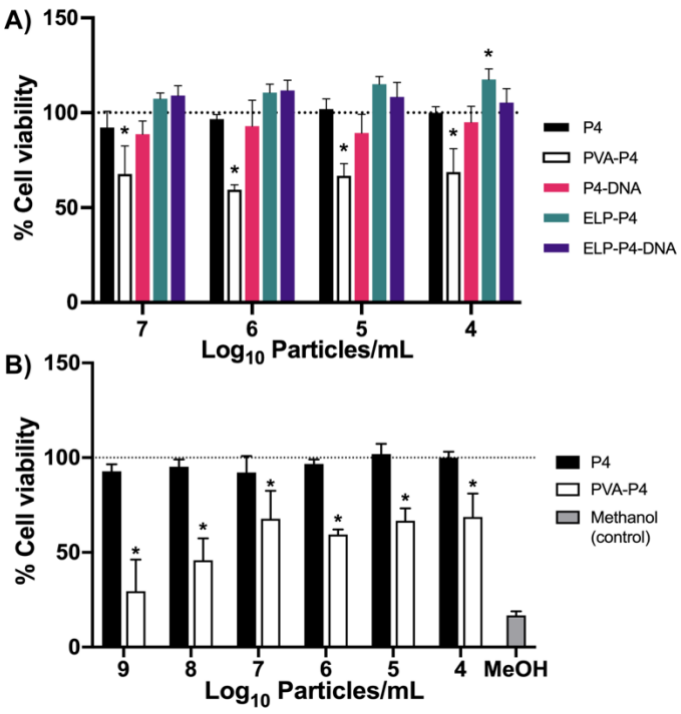


Figure 16. PVA-P4 nanoparticles are significantly more cytotoxic than are P4 nanoparticle or those P4 nanoparticles made with DNA and/or elastin. Cell viability, after 24 h, is reported as a function of exposure concentration to P4 (black), PVA-P4 (white), P4-DNA (red), and ELP- P4 with (purple) and without (green) DNA (A). Also included is cell viability at a larger range of P4 (black) and PVA-P4 (white) nanoparticle concentration (B). Data are presented as percent cellviability, which was determined by dividing the relative fluorescence units (RFU) of nanoparticle exposed cells by the RFU of cell only controls. Bars represent the mean and error bars represent the standard deviation of at least 4 independent wells. An asterisk (*) represents a significantly ($P < 0.05$) different mean cell viability. Significant differences were determined in panel A by an ANOVA followed by Tukey s method for nanoparticle exposed cells compared with the cell only control (dashed line), and in panel B by a two-tailed t-test used to compare P4 and PVA-P4 nanoparticle exposed cells within a given particle concentration.

CONCLUSION

In this study, various PLGAs with different chemical compositions were assessed for their ability to make stable nanoparticles for *in vivo* delivery and long-term storage (Table 1). When compared with PLGA compositions of different length, L:G ratios, and terminal functionality, P4 nanoparticles (24-38 kDa, 65:35, carboxyl-terminus) were found to be the most stable under storage and physiological conditions and were subsequently compared to the gold standard for PLGA nanoparticle synthesis using PVA stabilized fabrication methods (Fig. 3-5). Given the cytotoxicity of PVA formulations, a method for stabilizer-free PLGA nanoparticle synthesis is desired for therapeutic delivery (Fig. 16). With similar stability in water and PBS and little to no cytotoxicity, stabilizer-free P4 nanoparticles were determined to be superior to PVA formulations. Additionally, PVA-free P4 nanoparticles exhibit a highly negative surface charge (-63 mV), which may be beneficial in cellular uptake and signaling such as transdermal drug delivery (Fröhlich, 2012; Platel et al., 2016) and perhaps most importantly are stable at room temperature for at least 23 months which is ideal for storage, transport and use in austere and nonrefrigerated environments. By varying concentrations of PLGA:water we can partially control the size of P4 nanoparticles, which in turn can help control uptake by the desired cell population (Fig. S7-S8) (Shima et al., 2013). Furthermore, stable P4 nanoparticles were made with the addition of DNA demonstrating therapeutic capabilities for use in vaccines or gene delivery and were conjugated with ELP for increased immune tolerance and long-term stability (Fig. 4-5). Elastin-modified PLGA nanoparticles not only have a long shelf life, which can be important in storing and transport of vaccines, but also show promise in altering the interactions between delivery vehicles and cell surfaces *in vivo* (Cho et al., 2016). Future investigations will be conducted to increase tunability of the elastin-conjugated nanoparticles by varying the conjugated moiety, temperature, and ionic strength to control immune-cell targeting and release kinetics. Overall, a useful formulation was

developed consisting of stable, nontoxic P4 nanoparticles of different sizes that allows for the encapsulation of DNA and surface modification without the use of toxic stabilizers, for downstream application in drug and vaccine delivery and therapeutic treatment.

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CHAPTER 3: LONG LIVED NANOPARTICLES

INTRODUCTION

Vaccination is one of the best ways to prevent infectious disease outbreaks, but complete protection is typically only achieved after multiple immunizations (boosters). Therefore, development of vaccine delivery systems that can achieve a multi-dose vaccine efficiency within a single dose, has become a prominent field of research. Development of injectable, sustained release vaccines that gradually release immunogens in a controlled manner can reduce the need for multiple injections and improve patient compliance (Anderson & Shive, 2012; Jain, 2000; Mollica et al., 2008). To achieve sustained release within a single shot we have designed a variety of strategies for controlled release of vaccine constituents. These strategies include creation of 1) a suite of different sized nanoparticles that differentially degrade with time as a function of size and 2) a suite of particles, which may all be the same size, but differentially degrade due to changes in formulation (time, temperature, and concentration) (Fig. 1). In this chapter we will discuss the methods and results of producing long lived polymer NP delivery systems.

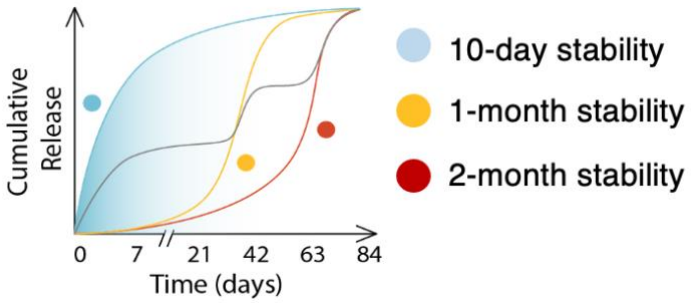


Figure 17

Visual representation of strategy 1 for creation of a one-shot vaccine delivery system. Here particles capable of differential degradation and hence differential release of vaccine substituents as a function of time are co-formulated together within a single dose.

Different attributes of the PLGA polymer can influence mechanical strength, control of hydrolysis, biodegradation rate, and swelling behavior of the polymer and its nanoparticles. Physical properties (size) and chemical properties (molecular weight, terminal group, and L:G ratio) can control the pharmacokinetic and biodegradable properties of particles at the micron scale. While much is understood how these factors control particle characteristics at the microscale, far less is understood how these factors control particle degradation and encapsulant release rates at the nanoscale. Here we investigate how size, chemical composition, and formulation conditions can affect the degradation rate of nanoscale particles. By doing so we've selected a number of PLGA's that allow for different L:G, molecular weight, and terminal groups to be tested (Table 1).

Name	Ratio (L:G)	Molecular Weight	Termination Group
P1 (P2191, Lot #066K1787)	50:50	40-75,000	Carboxylic acid terminated
P2 (P2191, Lot #MKBZ5057V)	50:50	30-40,000	Ester terminated
P3 (P2066, Lot #MKCD6328)	65:35	40-75,000	Ester terminated
P4 (RG 653, Lot #STBJ0282)	65:35	30,400	Carboxylic acid terminated
P5 (Degradex)	50:50	24-38,000	Ester terminated
P6 (RG 505, Lot #STBH9167)	50:50	54,000-69,000	Ester terminated
P7 (RG 752 S, Lot #BCBW2279)	75:25	12,500	Ester terminated
P8 (RG 502H, Lot #BCCC0979)	50:50	7-17,000	Carboxylic acid terminated

Table 4

An important behavior of NP delivery systems is the initial burst release and uncontrolled immunogen presence >50% within the first 24 hours. This has been extensively studied in micron particles, where investigators have found that burst release can be tuned by altering the PLGA monomer composition or by addition of stabilizers (Stromberg et al., 2021) (Fig. 2).

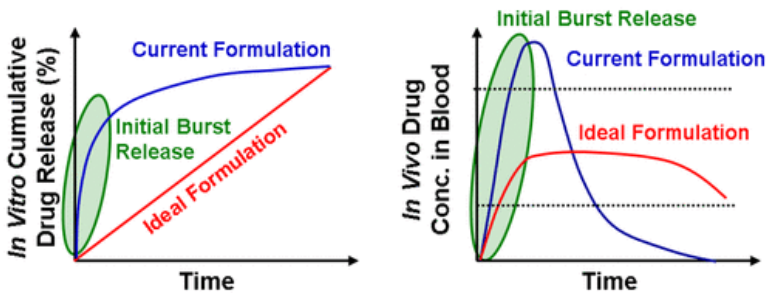


Figure 18

Initial burst release of drugs from PLGA microparticles are a major drawback to achieving sustained release drugs (Yoo & Won, 2020). The reduction of initial burst release is essential to creating sustained release NP delivery systems. *In vitro* drug release profiles are described above based on drug release vs time.

Our initial strategy for controlled release, was to create particles of difference sizes that differentially degrade with larger particles degrading slower and smaller degrading faster. This was observed for the microscale, whereby stir speed during solvent evaporation produced different sized particles with an inverse degradation relationship (larger = slower, smaller = faster)(Ravi et al., 2008). However, controlling nanoparticle size by varying stir speed (Fig. 3), was unfortunately ineffective for creating nanoparticles with reliably different particle sizes. We also found that the small differences in particle sizes achieved did not directly relate to differences in degradation time

(Ch 2) (e.g. the differences in degradation time were not substantial enough to mimic the time scale of prime and boost (7 to 21 days).)

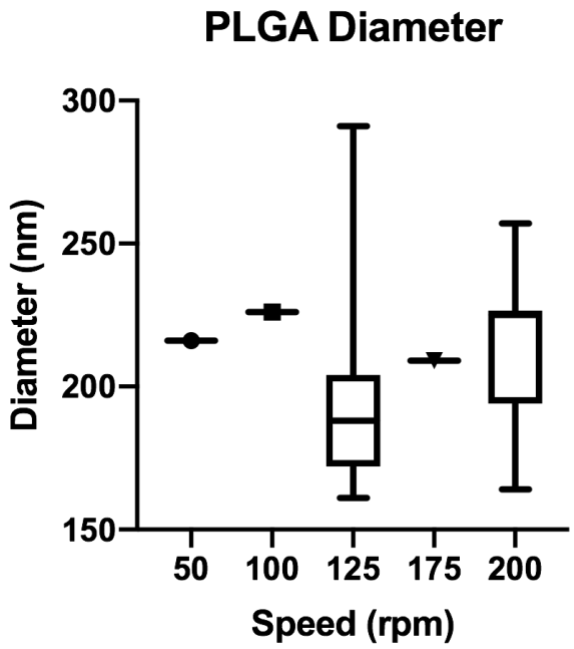


Figure 19

Stir speed does not reliably alter the size of P4 PLGA nanoparticles made by the solvent evaporation method.

The second strategy relies on creating a suite of differentially degrading particles due to changes in formulation (time, temperature, concentration), which may have the same particle size. Formulation conditions that produce particles that degrade within the timescales of a typical prime (<7 days) and boost (~21 and 28 days) are considered ideal time points. Through altered reaction conditions and different formulations, we aim to tune the degradation of PLGA NPs. Molecular weight, for example, is known to affect mechanical strength and, therefore, to degrade faster (Gentile et al., 2014; Panyam et al., 2002, 2003).

In addition to molecular weight, changes in L:G ratio can alter the degradation rate of PLGA particles in the micron scale (Amann et al., 2010; Faisant et al., 2002; Hirenkumar & Steven, 2012; Ramchandani & Robinson, 1998). Amann and colleagues demonstrated that increasing the lactic to glycolic stoichiometry in the PLGA monomer, decreased the initial burst release and created particles with longer stability (Fig. 4).

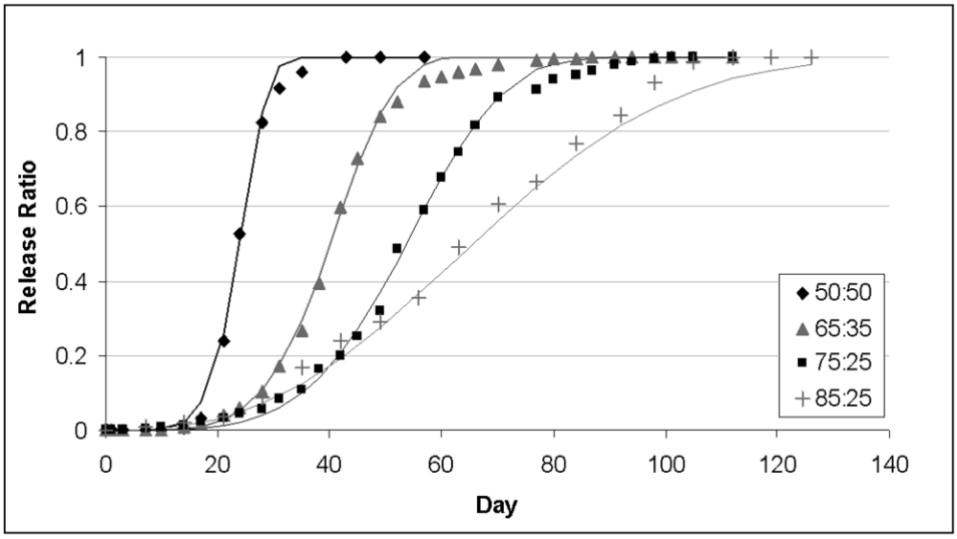


Figure 20

The in vivo release profile of ranging (50:50, 65:35, 75:35, and 85:15) L:G ratios in PLGA particle initial release periods, typically followed by rapid drug release. In the micron particle literature, reduction of initial release rate is controlled through L:G (Makadia & Siegel, 2011).

Another method to create particles with differential degradation is by altering the formulation conditions (e.g. temperature). For example, high temperature is found to increase the thermal motion of polymer long chains, which changes the fluidity of self-assembly and ultimately packing density (Chai et al., 2017). Different mobility of the polymer molecules in the matrix can lead to stronger packing density of the outer shell of the particle. It is presumed that

this change in the outer shell stability then decreases the ability for ester hydrolysis of the PLGA particle in whole.

Both cellular uptake and endosomal release are critical for DNA to be a potent vaccine (Bolhassani & Yazdi, 2009). After uptake by the cell, the cargo must avoid endo/lysosomal degradation and enter the cytoplasm through "endosomal escape," achieved by particle swelling and burst(Freeman et al., 2013). It has been shown that charge (e.g. cationic surface functionalization) can alter the endosomal escape of particles and also serve as mild adjuvants (e.g. they increase the effectiveness of vaccines by stimulating the immune system). Ranging from lipids, polymers, to surfactants, many papers have investigated DOTAP, CTAB, DOTMA, DDA, and more for their adjuvant potential and anionic interactions (e.g. DNA condensation and PLGA packing) (Felgner et al., 1987; Singh et al., 2000). Here, we have focused on the cationic surfactant, Cetyltrimethylammonium bromide (CTAB). While previous studies have shown that particles with CTAB significantly increased expression of CD80, CD86 and MHC II on the surface of dendritic cells in lymph nodes, (Liu, Ni, et al., 2020; Liu, Yu, et al., 2020; Zhang et al., 2020) we utilized CTAB to create polymer layers within particles and to alter the stability and net charge of our PLGA formulations.

Finally, the temperature at which particle stability is stored and formulated greatly alters their apparent stability and burst release. For example, it is common to characterize said formulations at refrigerated and room temperature conditions, and not biological conditions (e.g. 37^{o}C). As a result, these investigations nearly always overreport the stability of their particles. To mimic these biological environments, we observed stability within endosomal and interstitial environments at different pH levels, pH 5.6 and 7.4 and at physiological temperature.

We investigated each of these approaches to create particles with different degradation rates. As we showed in chapter 2, particles of different sizes can be created by controlling the stoichiometry of PLGA to water (Table 3, Chapter 2). But the difference in their degradation is not significant enough to create a suite of differentially degrading particles (e.g. it does not differ by more than 5-7 days). Thus, we narrowed down our selection to strategy 2 (e.g. nanoparticles with roughly the same size that by virtue of different structure or formulation, degrade at short-, medium-, and long-time frames). In this chapter we will discuss the methods and results of producing a suite of short- and long-lived polymer NP delivery systems, by control of their starting concentration, PLGA polymer formulations (mixtures), reaction temperature and charge.

MATERIALS AND METHODS

Formulation and Solvent Evaporation

PLGA NPs were prepared by solvent evaporation method, previously explained in Chapter 2. Most typically, 25 mg of PLGA was dissolved in 2.5 mL of acetonitrile. This organic polymer solution was added dropwise into a 7.5 mL filtered nanopure water solution, while stirring at room temperature. If formulation included CTAB, 0.5% wt/vol CTAB was added to the aqueous solution containing nanopure water (elaborated in following paragraph). If the formulation included alternative concentrations of PLGA, less or more was added to 2.5 ml acetonitrile. The mixture was left to stir for 24 hours at room temperature to allow for the organic solvent to evaporation and for the NPs to form. If the formulation included alternative temperatures (37^{o}C) the solvent evaporation method was at elevated temperatures. The reaction solution was then dialyzed overnight against 2 L nanopure water (with changes of nanopure water after 2 h) in a 20,000 kDa molecular weight cutoff Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific). Each NP

formulation was examined by dynamic light scattering and zeta potential for size and charge. Day 0 of each measurement was PLGA NPs as formulation (e.g. within aqueous solution). After initial measurements, the formulations were placed in 1X PBS pH 7.4 and stored in a 37^{o}C standing incubator for remaining stability studies (Day 1 forward).

DNA Addition: Addition and Encapsulation

For NP made with plasmid DNA, varying formulations were produced. Two methods for DNA protection by PLGA particles were investigated: addition versus encapsulation. In the literature many methods of DNA delivery are through surface addition (post formulation of PLGA particles incubation of DNA). Here for example, the PLGA delivery system is positively charged and electrostatically interacts with the negatively charged DNA (e.g. phosphate groups on DNA). Here following formulation of CTAB-PLGA (e.g. P4 or P7) the particles were incubated with ranging concentrations of DNA to allow for surface bound delivery.

Second method is through encapsulation. Here the DNA, large polyanion, must be first condensed into a smaller package prior to encapsulation within the PLGA particle. This increases the encapsulation efficiency within partially negative PLGA. This condensing process is done prior to the addition of PLGA during solvent evaporation method. Typically, 0.5% wt/vol CTAB is added to 12.5 ng/ul of DNA for 30 minutes prior to addition of dropwise organic solvent with PLGA.

Formulation to alter lifetime of nanoparticle: Varying concentration PLGA

By altering the concentration of PLGA (12.5 mg/ml to 100 mg/ml) we found the nanoparticles to be smaller in size (100 nm, 12.5 mg/ml), but more stable (7 days to 14 days) compared to our standard reaction of 25 mg/ml. This formulation will be mentioned again for one

of our selected suites of particles. By increasing the concentration of PLGA we found the self-assembly process to be unstable. The NPs were much larger and more polydisperse (data of polydisperse 100 mg). The increased concentration of PLGA led to unstable particles, aggregated, and fell out of solution in PBS (< 1 day stability). For medium-lived formulation "half concentration P4" were made with half the total PLGA concentration typically added, 12.5 mg/ml of PLGA. Half concentration P4 NPs around 110 nm and stable up to 38 days. After 38 days the particles began to increase in size by DLS to 140 nm. table

Name	Ratio (L:G)	Molecular Weight	Termination Group
P1	50:50	40-75,000	Carboxylic acid terminated
P2	50:50	30-40,000	Ester terminated
P3	65:35	40-75,000	Ester terminated
P4	65:35	30,400	Carboxylic acid terminated
P5	50:50	24-38,000	Ester terminated
P6	50:50	54,000-69,000	Ester terminated
P7	75:25	12,500	Ester terminated
P8	50:50	7-17,000	Carboxylic acid terminated

Table 5

PLGA polymers (P1-P8) with varying lactic acid to glycolic acid (L:G) ratios, molecular weight, and terminal groups.

Temperature Increase P4

For short-lived formulation, P4 was made as solvent evaporation method previously stated, a total of 25 mg/ml of P4 (65:35, 24-38 kDa, carboxylic terminated) at increased temperatures (30ºC). Temperature increased P4 NPs were 130 nm (-60 mV) and had stability up to 17 days. After day 17 the particles began to increase in size by dynamic light scattering to ~600 nm.

Layer by layer (LbL) polymer nanoparticles

To create layer by layer nanoparticle formulations we investigated adding alternating charge materials to build up the nanoparticles surface. This was also done similarly to solvent evaporation method mentioned above. First layer (core, red) was formulated by taking vial 1 containing 7.5 ml distilled water and stirring at room temperature. Vial 2 containing 25 mg of P4 PLGA polymer solution and dissolving in 2.5 ml of acetonitrile. Vial 2 was added dropwise to vial 1 and allowed for solvent evaporation to occur for the following 24 h at room temperature. After acetonitrile had evaporated off, a vial containing P4-P7 (12.5 mg/ml of each PLGA polymer) in 2.5 ml of acetonitrile was added to the initial vial 1 (room temperature for 24 hours). Lastly, the final layer of P4 polymer was added and allowed for an additional 24 hours. Between each layer addition, size and charge was recorded to ensure increase in particle size and variation in surface charge.

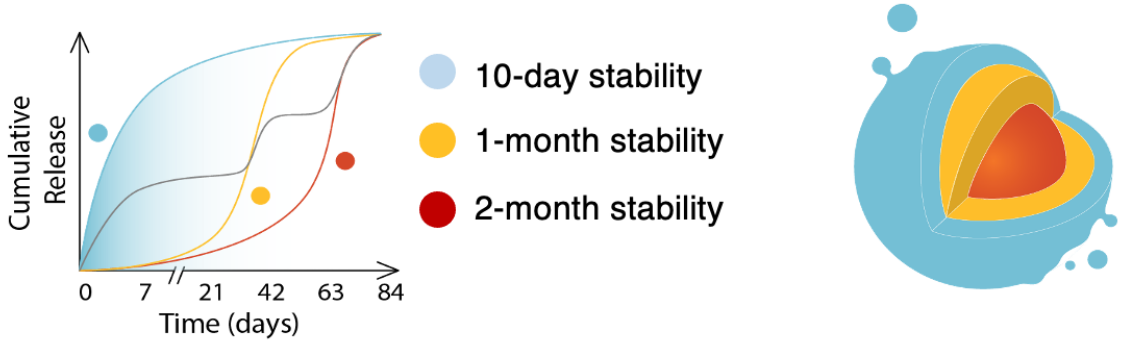


Figure 21

Layer by layer (LbL) nanoparticle formulation goal of sustained release sequential degradation of layers of delivery system that allow for mimic of primary and booster vaccination.

Nanoparticle Characterization: Nanoparticle size, zeta potential and stability

PLGA nanoparticles were characterized using dynamic light scattering for size and zeta potential as previously described (Stromberg et al., 2021). Size standards were Nanosphere polymer microspheres in water from Thermo Fisher Scientific. Vendor reported size values (NIST traceable) for 100 nm and 200 nm beads are as follows: mean diameter 100 nm ± 3 nm, size distribution 7.8 nm, standard deviation 7.8%, and 203 nm ± 5nm, size distribution 5.3 nm, and standard deviation 2.6%. Standards were diluted in 0.2 μm filtered nanopure water. Measurements were performed at 25°C in plastic disposable cuvettes. Size and zeta for day 0 was measured in water and taken at room temperature. Size and zeta for day 1 and following days were taken in 1X PBS solution (pH 5.6 or pH 7.4) for size and 0.1X PBS for zeta. Day 1 and following day samples were stored in 37ºC, although characterization measurements were made at 25ºC.

Determining loading efficiency of DNA

To measure release of PLGA NPs DNA was fluorescently labeled (Mirus Label IT kit, MIR #7100, MFP488) (Fig. 6) prior to encapsulation within PLGA NPs. After the solvent evaporation method, 200 ul of PLGA sample was removed and added to a 2 ml microcentrifuge tube. The microcentrifuge tube was spun at 10,000G for 5 minutes to remove NPs. Supernatant was removed (100 ul) and additional CTAB (50 mM, 100 ul) was added. Thirty-minute incubation of supernatant and CTAB is required prior to addition of methanol (200 ul) and sodium chloride (1 M, 50 ul). An hour incubation was required after addition of methanol and sodium chloride, and the solution was then vortexed, and an emission scan was taken (501: 515-550) and emission at 526 nm was recorded for DNA release and compared to a standard curve (0, 0.06, 0.125, 0.25, 0.5, 1 ng/ul).

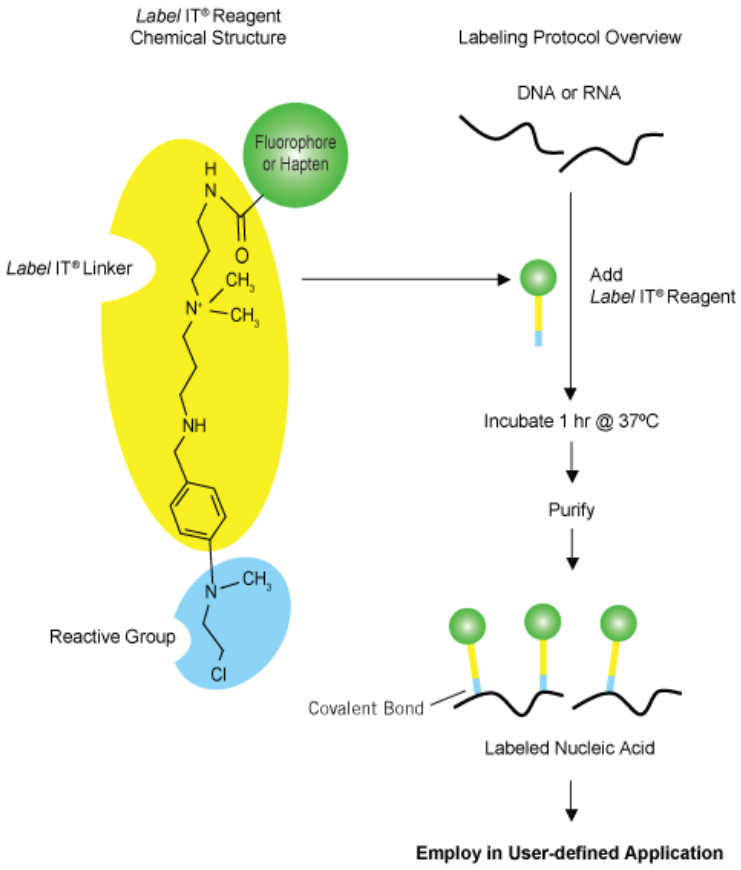


Figure 22

Label IT nucleic acid labeling kit consists of three components. The label (fluorophore), linker (facilitates electrostatic interactions with alkylating group) and covalently attaches to any reactive heteroatom within the nucleic acid. Details about Mirus kit can be found on website.

Measuring stability and degradation of nanoparticles

Nanoparticles were placed in varying pH environments to replicate the biological environments of endosomal pH or interstitial pH (Evans et al., 2013). Low and neutral pH PBS buffer was made with sodium phosphate dibasic and monobasic solutions. To 800 mL of distilled water was added 20.214 g of Na_2HPO_4-$7H_2O$ to the solution and 3.394 g of $NaH_2PO_4H_2O$ to the solution. Achieved desired pH by HCl or NaOH and added distilled water until volume is 1 L. Final solution had a molarity of 0.1 M and pH 7.4 or 5.6.

RESULTS

Suite of particles with different degradation rates

Our strategies to create sequential vaccine delivery consist of 1) different sized nanoparticles that differentially degrade as a function of size and 2) a suite of differentially degrading particles due to changes in formulation (time, temperature, concentration), which may have the same particle size. We had two strategies to create particles of different size (by altering stir speed or stoichiometry). While, stir speed did not reliably alter the particle sizes, Figure 3, changing the stoichiometry of PLGA to water did change the particle size, but their difference in degradation were not substantial enough to mimic a prime and boost (in contrast to that seen for micron particles). Below we present the results from our second method where we were able to successfully create short-, medium- and long-lived particles (7, 30, and 60 day) that mimic the prime and boost response (e.g. 21-28 days) in a typical vaccination timeline (Fig. 7).

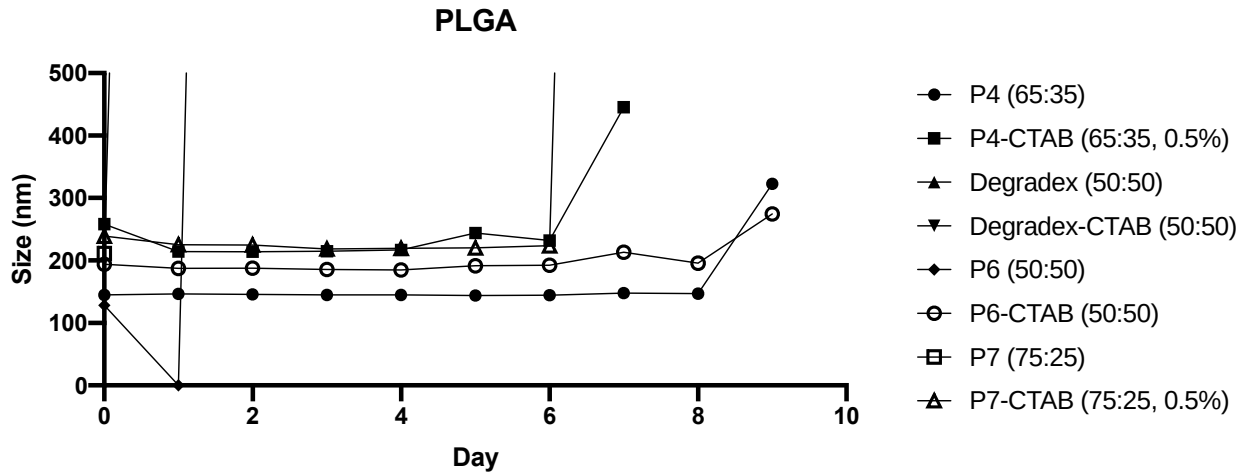


Figure 23

Stability and degradation for several PLGA variants assessed. All nanoparticles were made at room temperature and placed in 1X PBS pH 7.4 on day 1 and stored at 37ºC.

The second strategy to create a suite of long-lived particles, was to alter the formulation conditions. We created short lived particles with our original P4 PLGA formulation, medium lived particles by decreasing the concentration of P4 PLGA by half or by increasing temperature during formulation, and long-lived particles by mixing different PLGA polymers during formulation (P4-P7). All PLGA NPs were prepared by solvent evaporation method, as previously described. Formulations were written as described, total PLGA concentration of 25 mg/ml. Poly (lactic co glycolic) (PLGA) (Sigma #719862) was added to a scintillation vial with acetonitrile to completely dissolve polymer. Once polymer was completely dissolved in organic solvent it was added dropwise to nanopure water while gently stirring.

As shown in Figure 7, we produced several different PLGA NPs that differentially degrade. We then narrowed down the best options for our primary and boost vaccination effect. We note that beyond the formulations above, we can alter the stability of those formulations by addition of CTAB. Generally, addition of CTAB results in particles degrading faster than their

constituent PLGA. For example, when CTAB is added to P4-PLGA the particles were 220 nm in size and stable for 7-9 days, versus P4-PLGA alone which was 140 nm in size and stable for 10-14 days. Like most of our particles, these CTAB modified PLGA particles also demonstrate differential degradation as a function of pH. For example (Fig. 8), CTAB-P4 degrades in 20 days at 7.4 pH and 15 days in 5.6 pH, similar trends are observed for other PLGA formulations as pH 5.6 leads to a faster degradation of the lactic and glycolic acid components.

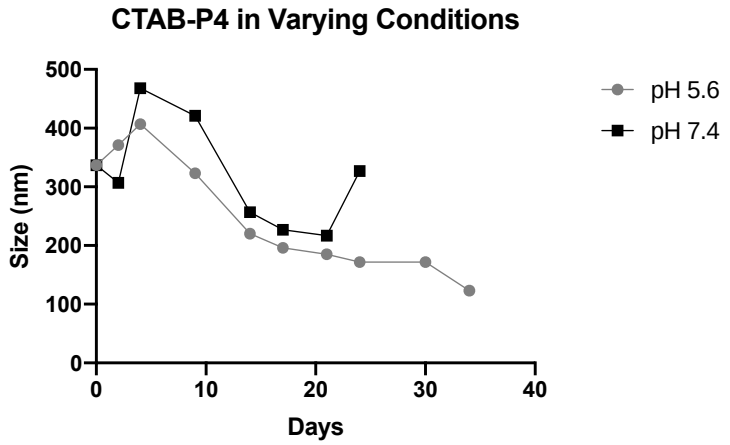


Figure 24

Stability of CTAB-P4 was observed in varying biological environments. Samples were placed in pH 5.6 to replicate endosomal environment and pH 7.4 for interstitial fluid to observe the stability and influence of particles. Nanoparticles were placed in 1X PBS solution and stored at 37^{O}C for up to 35 days. *Day 0 was recorded at room temperature in nanopure water.

Selection of primary and secondary (booster) vaccination delivery systems

For ease, we start by describing the short-lived formulation, P4 PLGA NP, as a reference point. P4 (25 mg/ml, 7-day stability, 200 nm) has the shortest stability mimicking the primary vaccination (Fig. 9).

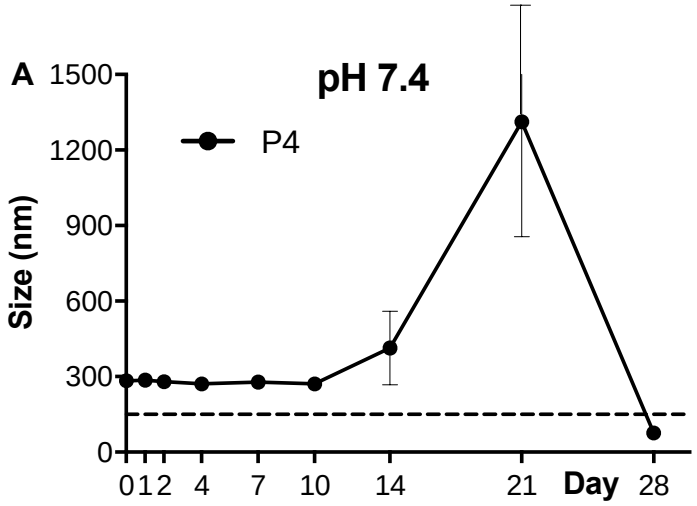


Figure 25

Short-lived formulation, P4 PLGA NP, stability in pH 7.4 1X PBS.

Next, we describe the medium lived formulation of half-P4 (12.5 mg/ml, 30-day stability, 100 nm) (Fig 12) vs. increasing the reaction temperature for P4 emulsification (25 mg/ml, 35^{o}C, 15-day stability, 130 nm) that create particles that may allow for a primary booster effect. By increasing the temperature during solvent evaporation method, we see an increase in stability as well (Fig. 9 compared to Fig. 10). This is likely due to the change in self-assembly thermodynamics that occur at increased temperatures (Gerstner 2018, Cao 2019). By increasing the temperature from room temperature to 30-35^{o}C for P4 emulsification we see the stability increase from 9 days to 17 days (Fig. 10). Whereas P4-P7 formulated at RT and at 40^{o}C saw a decrease in stability when temperature was increased, this may be due to P7 not incorporating with the P4 polymer at increased temperatures (Fig. 11). P7 alone is not very stable, stability is less than 10 days, and could be the contributing factor to decreased stability, which may arise from the lower molecular weight and ester termination, creating weaker packing. Ester terminated PLGA will be more hydrophobic than the acid terminated, thus you can expect more

attraction between PLGA molecules than with water. P4-P7 synthesized at room temperature had stability up to 40 days whereas P4-P7 synthesized at 40°C is stable only to 20 days.

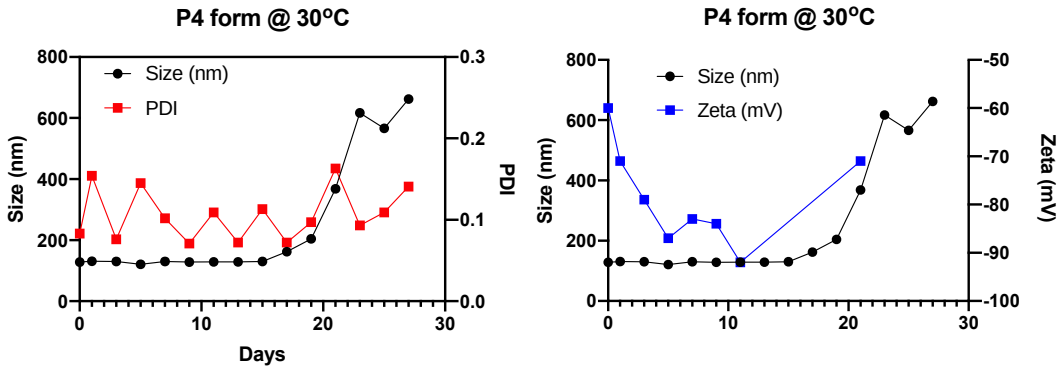


Figure 26

Stability of P4 nanoparticles formulated at 30°C during solvent evaporation method. Size was recorded for 30 days, nanoparticles lost integrity around day 20. P4 at increased.

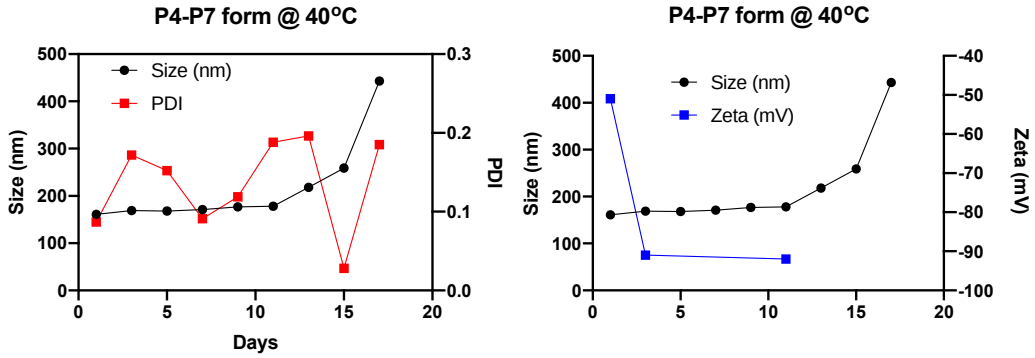


Figure 27

Stability of P4-P7 for size and zeta potential was recorded. Samples were made at 40°C and stored at 1X PBS pH 7.4 at 37°C. P4-P7 was more stable at room temperature formulation.

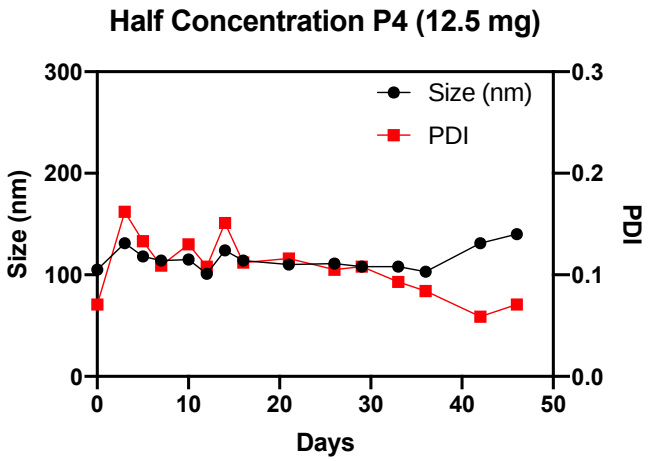


Figure 28

Stability of P4 at half concentration was recorded for 50 days, taken at pH 7.4 1X PBS stored at 37^{o}C. Nanoparticles began to lose their integrity around day 35.

Lastly, for long-lived formulation a mixture of P4-P7 (60-day stability, 230 nm) was created with half P4 (65:35, 24-38 kDa, carboxylic terminated, 12.5 mg/mL) and half P7 (75:25, ester terminated, 12.5 mg/mL); using the solvent evaporation method detailed in the methods section. P4-P7 NPs were 230 nm and stable up to 52 days (Fig. 13).

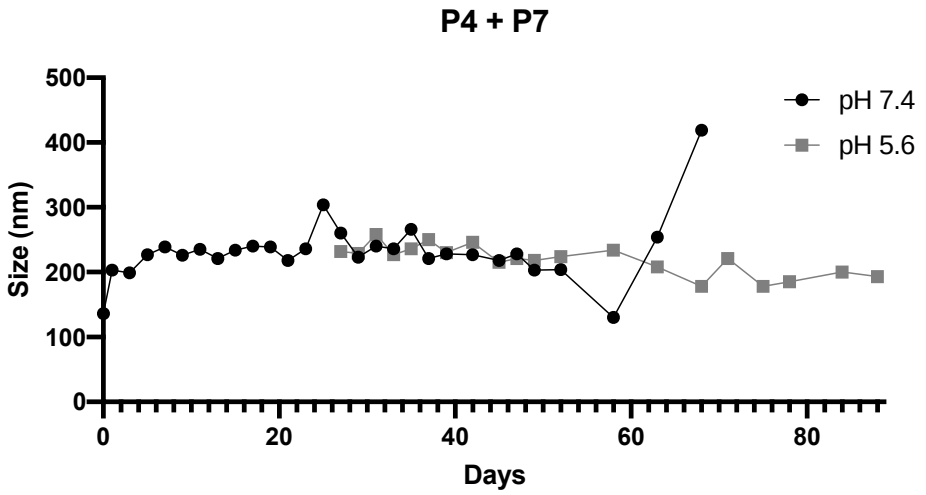


Figure 29

Stability of P4-P7 nanoparticles in varying pH environments. Nanoparticles were stored at 37ºC.

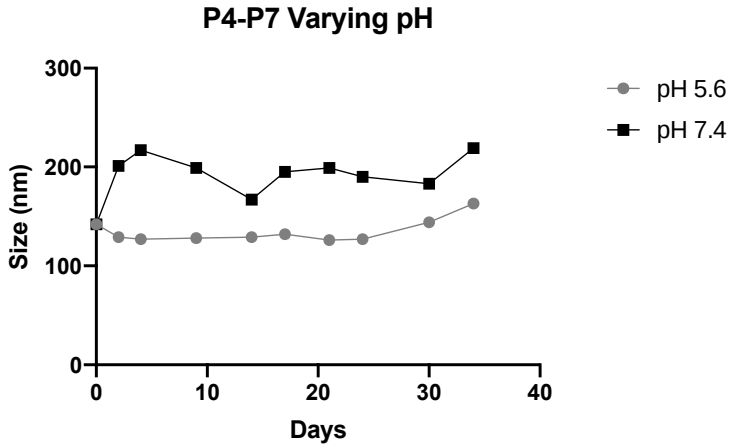


Figure 30

Stability of P4-P7 nanoparticles was recorded for 35 days in pH 5.6 and 7.4 mimicking biological environments.

These long-lived P4-P7 particles appear to be individual particles composed of both P4 and P7 and not mixtures of individual P4 and P7 particles. For example, P4 by itself creates particles of 200 nm that degrade in 7 days (see above, Fig. 9). Likewise, P7 by itself produces particles of 200 nm that are unstable in PBS after 24 hours. Like all our particles, the exact nature of the

decomposition of the particles upon degradation is unknown and is being analyzed by changes in pH and atomic force microscopy but may result from surface degradation and then bulk aggregation and flocculation of the degrading particles. The long-lived P4-P7 particles were then manipulated to see the difference in degradation when cationic component is added (Fig. 15) and comparing the addition to that of P4-P7 alone (Fig. 16). We found that by adding CTAB into our formulation the degradation of the particles decreased, showing the capability to increase or decrease the release based off application necessary.

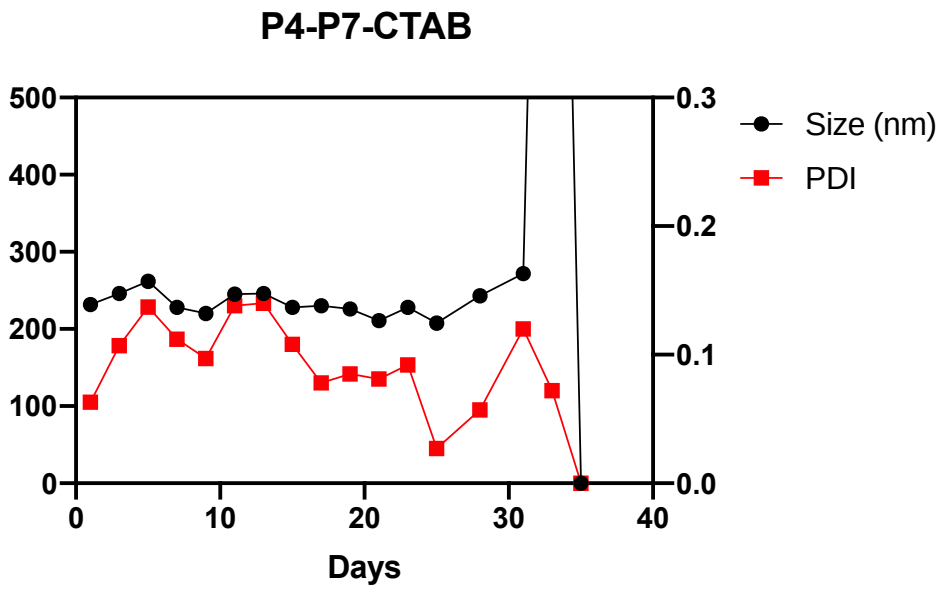


Figure 31

Stability of P4-P7-CTAB over 40 days in 1X PBS pH 7.4 stored at 37ºC. Nanoparticles were stable up to day 30.

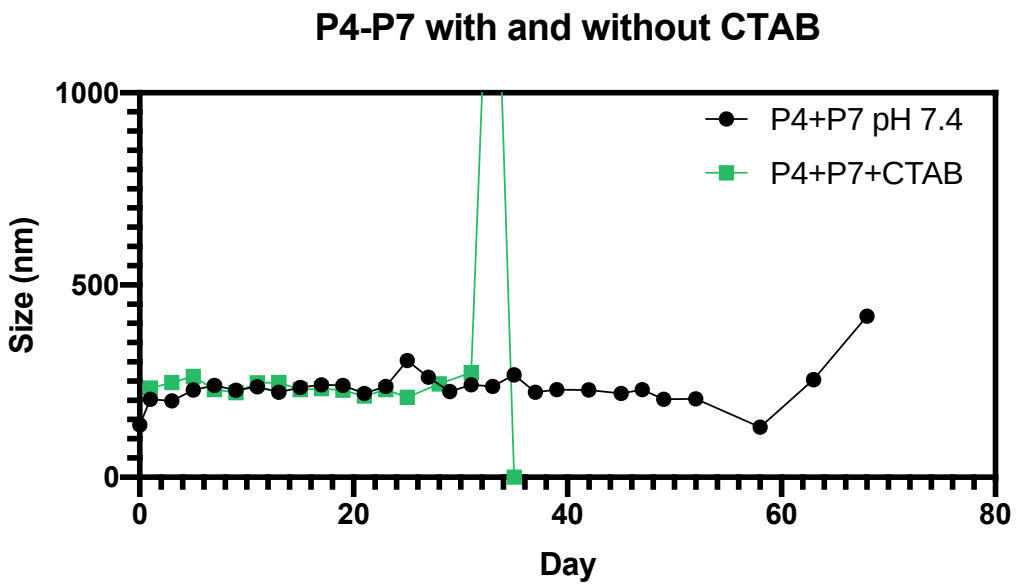


Figure 32

Comparative stability data of P4-P7 with CTAB and without CTAB added. By adding the cationic surfactant, we see decreased stability.

Measuring encapsulation of DNA within delivery systems

Again, one drawback of solvent evaporation method is poor encapsulation efficiency of DNA. It is found that by encapsulating the negatively charged DNA within the anionic PLGA polymer there was some diffusion or partition from the two phases that would discourage the encapsulation. We've used CTAB to condense our DNA prior to encapsulation and found that CTAB interferes with our fluorescent signal when measuring release. As mentioned in methods, we created an assay that removes CTAB aggregates by increasing the CMC. Prior to creating our assay for CTAB removal we used a Qubit with 1X Quant-IT kit that showed extremely high DNA encapsulation for our P4-CTAB-DNA particles. This was a trend in the literature that stated high

encapsulation with CTAB with a direct correlation to increase of CTAB. Encapsulated DNA release is currently being investigated, to correlate with particle degradation.

Sample	*Day 1*			*Day 5*		
	Size (nm)	Zeta (mV)	% DNA encapsulated	Size (nm)	Zeta (mV)	% DNA encapsulated
P4	193	-13	N/A	200	-51	N/A
P4+CTAB	343	77	N/A	654	87	N/A
P4+CTAB+DNA	418	86	93	395	86	95

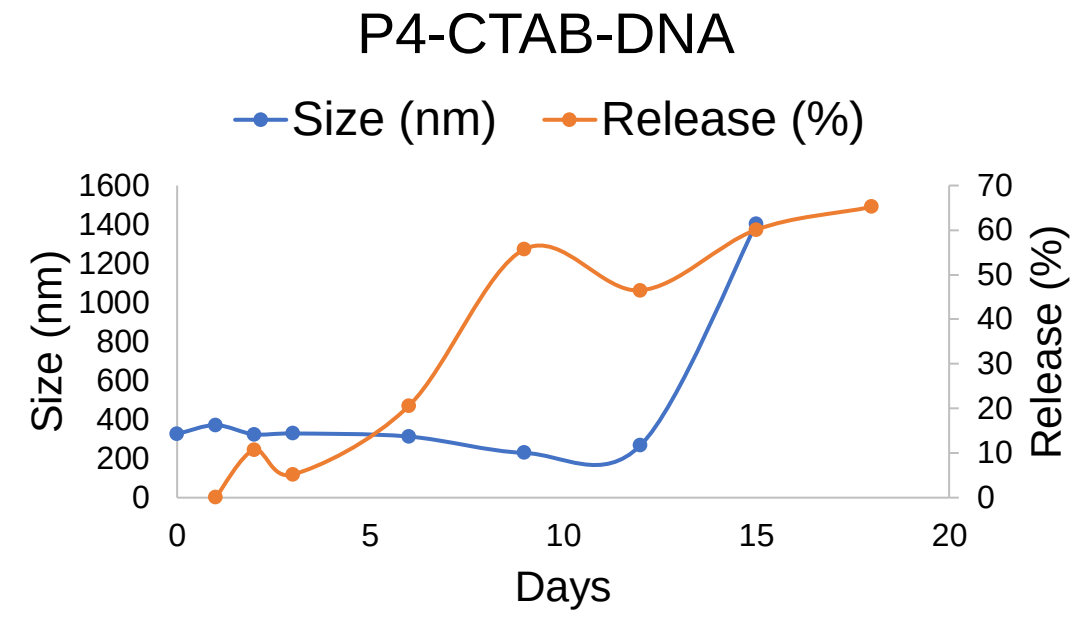
Table 6

After determining the CTAB interference with the fluorescent emission signal and measuring the DNA release of the particles using our CTAB removal assay we found encapsulation to be around 50-60% (Table 3 and 4). These P4-CTAB-DNA particles were stable up to 15 days and size remained around 300 nm.

Sample	**Day 0**		
	Size (nm)	**Zeta (mV)**	**% DNA Encapsulated**
P4-CTAB-DNA	292	75	67

Table 7

Showing the P4-CTAB-DNA encapsulation efficiency when using the fluorescently labeled DNA prior to nanoparticle encapsulation (solvent evaporation method) and MeOH/NaCl assay detection method. P4-CTAB-DNA found to have 67% encapsulation of pDNA.



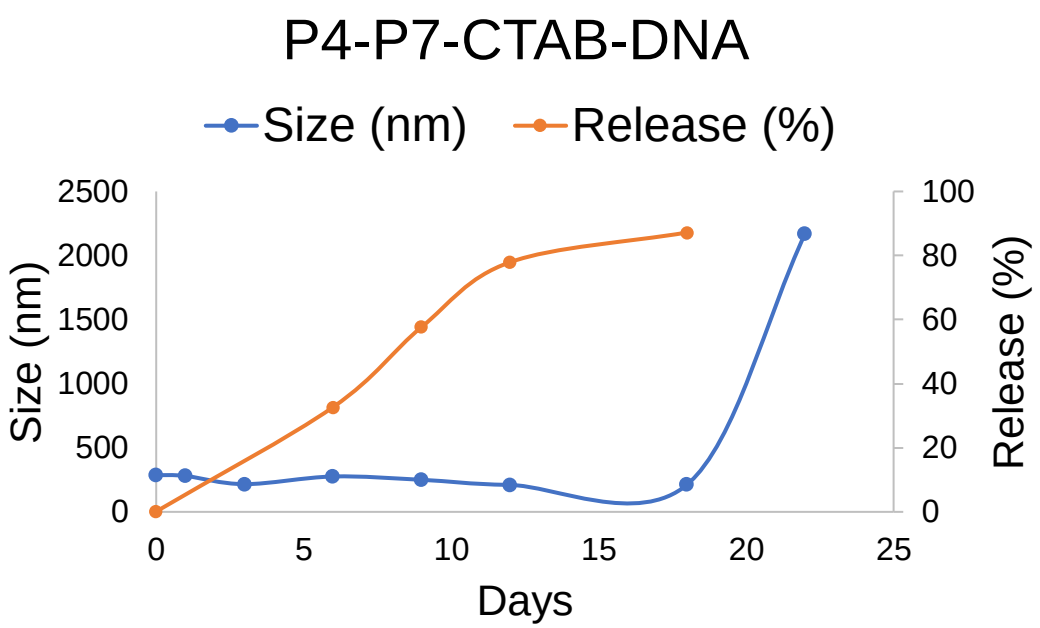


Figure 33

P4-CTAB-DNA release was measured over 18-day period, release was seen gradual and comparable to that of P4-CTAB-DNA particle degradation. Also observed P4-P7-CTAB-DNA and saw a correlation with particle degradation and DNA release.

CONCLUSION

By creating a suite of particles with differential degradation we can achieve a primary and booster vaccination effect within a single dose. The goal to inject three different delivery system formulations that have 7-, 15-, 30-, and 60-day release degradations can allow sustained release of vaccine components. Our short-lived particle, P4 PLGA NP, will create the primary booster effect with a 7-day degradation period. Our medium lived particles, P4 at increased temperature and half concentration P4, allow for a booster shot effect with 15–30-day degradation. Lastly, our long-lived particle, P4-P7, to give a final booster effect and results in the final administration to achieve greatest immune response. These particles are roughly the same size on the nanoscale 200-300 nm that create a monodisperse solution and each degrade faster in endosomal environments (pH 5.6). This gives the highest confidence that when the delivery systems are engulfed by endosomal uptake that within the endosome the particle will begin to release the pDNA cargo in the corresponding delivery location.

The next steps in producing these PLGA NP sustained release one-shot delivery systems are to monitor the release effect of said particles. Monitoring the release of DNA from the PLGA deliver systems will show if particle degradation corresponds directly to nanoparticle degradation. This question in the literature hasn't been directly addressed comparing benchtop methods of degradation to that *in-vivo*.

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