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Cross-platform comparison of therapeutic delivery from multilamellar lipid-coated polymer nanoparticles

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Abstract

Significant efforts have been invested in finding a delivery system that can encapsulate and deliver therapeutics. Core-shell polymer-lipid hybrid nanoparticles have been studied as a promising platform because of their mechanical stability, narrow size distribution, biocompatibility, and ability to co-deliver diverse drugs. Here, we designed novel core-shell nanoparticles based on a poly(lactic-co-glycolic acid) (PLGA) core and multilamellar lipid shell, where the lipid bilayers are crosslinked between the two adjacent bilayers (PLGA-ICMV). The cross-platform performance of our nanoparticles to other polymer-lipid hybrid platforms was examined, including physicochemical characteristics, ability to encapsulate a variety of therapeutics, biocompatibility, and functionality as a vaccine delivery platform. We observed differential abilities of nanoparticle systems to encapsulate distinct pharmaceuticals, which suggests careful consideration of the platform chosen depending on the therapeutic agent and desired function. Our novel PLGA-ICMV platform demonstrated great potential in stably encapsulating water-soluble agents and therefore is an attractive platform for therapeutic delivery.

Graphical Abstract

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Novel nanoparticles consisting of a PLGA core surrounded by an interbilayer-crosslinked multilamellar vesicle (PLGA-ICMVs) are designed and evaluated with cross-comparison to bare PLGA particles and PLGA-liposomes. This integrated platform combines attributes of solid nanoparticles and multilamellar lipid vesicles, such as colloidal stability, high encapsulation of diverse drugs, and biocompatibility, and shows promise as an immunogenic vaccine delivery system.

Keywords

polymer-lipid nanoparticles; drug delivery; biomaterials; vaccine delivery

1. Introduction

Nanoparticles (NPs) have been used extensively for drug delivery applications. Among their benefits are encapsulation of poorly water-soluble drugs, lower immunogenicity, sustained release of the drug, and targeted delivery [1]. Lipid and polymeric nanocarriers are the most common nanomaterial platforms. Lipids are amphiphilic molecules that are biocompatible and biodegradable, and demonstrate additional advantages because they are easily synthesized in a tunable way and allow encapsulation of both water-soluble entities and hydrophobic molecules in their bilayers. However, their structural stability is dependent on the environment conditions and can be altered due to changes in pH, ionic strength, and temperature [2]. Polymeric NPs are another type of drug delivery system. They allow size distribution control, high surface to mass ratio, sustained release of drugs, and mechanical stability. However, they tend to have low drug loading and burst release their loaded drug [3].

At the intersection of these two platforms are polymeric-lipid core-shell nanoparticles, which are an emerging drug delivery platform for diverse biologics and small molecule drugs [4]. These nanoparticles are attractive vehicles because they exhibit the characteristics of both the polymeric core and the lipid shell. The polymer core provides a high surface area, good stability, biodegradability depending on the polymer used, and defined morphology. The lipid shell is biocompatible, facilitates interactions with many molecules due to its amphiphilic nature, can be modified for targeted delivery, and provides steric stabilization for the core-shell complex [4]. A common core polymer is poly lactic-co-glycolic acid (PLGA) [6] that has been shown to deliver small molecule drugs [7] and proteins [8]. PLGA is biodegradable, biocompatible, easily synthesized in different NP size ranges, able to load many biological molecules, and is FDA approved [9]. Therefore, synthesis of hybrid NPs that are composed of a PLGA polymeric core and lipid shell exploits both of their advantages.

Many research efforts have been performed to further develop this platform and to explore their delivery performance. Bershteyn et al. [10] showed that the core-shell structure could be manipulated according to the lipid envelope composition. Incorporation of lipid-polyethylene glycol (PEG) conjugates in the lipid shell induced the formation of “flower” shape morphologies where the lipids form petals over the polymeric core. However, when

the lipid shell was composed of the zwitterionic lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), it formed multilamellar, onion-like bilayers on the surface of the polymeric core. Lipid mixtures of negatively charged and zwitterionic lipids exhibited morphology of one lipid bilayer on the surface of the polymeric core. PLGA-lipid NP hybrids were shown to be able to load proteins, cancer therapeutics, and DNA efficiently for delivery applications [11]. Moon et. al. [16] conjugated Vivax Malaria Protein 001 (VMP001) antigen on the surface of the lipid-polymer NPs and together with the immunostimulatory molecule monophosphoryl lipid A (MPLA) they elicited durable humoral immune responses against the VMP001 antigen. Zhong et. al. [12] demonstrated that PLGA-lipid hybrids are able to transfect luciferase plasmid DNA to cells. They showed that the method of DNA loading, either adsorbed on the outer surface or encapsulated within the NP could affect the uptake of the NPs by the cells, as well as the release of DNA. Recently, Moon et al. [18] developed novel liposome-based nanoparticles for protein therapeutic delivery that are based on multilamellar liposomes that are crosslinked between two adjacent lipid bilayers and so-called named interbilayer-crosslinked multilamellar vesicles (ICMVs). These ICMVs exhibited stable entrapment of protein antigens in their vesicle core and adjuvant molecules in their bilayer membrane, which formed a potent vaccine that elicited strong T-cell and antibody responses.

Inspired by both PLGA-lipid hybrids and ICMVs as biological molecule carrier platforms, we designed novel polymer-lipid hybrid NPs composed of a PLGA core and bilayer lipid shell that is crosslinked between two adjacent lipid bilayers (PLGA-ICMVs). We hypothesized that this novel platform will provide better colloidal stability compared to other PLGA and liposome-based systems and improved encapsulation because of the multiple lipid bilayers comprising the shell. We synthesized PLGA-ICMVs and directly compared the characteristics of it to other PLGA-based delivery platforms. We characterized the PLGA-ICMVs using cryogenic-transmission electron microscopy (cryo-TEM), dynamic light scattering (DLS), zeta-potential, and loaded different biological molecules (small hydrophobic and hydrophilic small molecule drugs, proteins, and plasmid DNA) and determined the encapsulation ability of our novel platform compared to PLGA-liposome core-shell particles and PLGA NPs. Finally, we investigated the functionality of these NP systems as a vaccine platform.

2. Material and Methods

2.1 Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (sodium salt) (MPB) were purchased from Avanti Polar Lipids Alabaster, AL, USA). PLGA (50:50 ester terminated, MW 52–54 kDa, inherent viscosity 0.55–0.75dL/g) was purchased from Lactel Absorbable Polymers. 1,4-Dithiothreitol (DTT), bovine serum albumin (BSA), and 3'-Azido-3'-deoxythymidine were purchased from Sigma. Etravirine (ETR) was a generous gift from I. Suydam, the department of Chemistry, Seattle University. Endotoxin-free Ovalbumin was obtained from Invivogen (San Diego, CA). EGFP was purchased from Aldevron, ND, USA and was amplified by *Escheria coli* cells (Gibco Life Science Technologies, Grand Island,

NY). Then, the plasmid was purified and extracted by a plasmid Giga kit (Qiagen, Valencia, CA). TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program.

2.2. Fabrication of Nanoparticles

Bare PLGA NPs (empty or with the hydrophobic agent ETR) were fabricated by using the single emulsion/solvent evaporation method. PLGA (with or without 1mg of ETR) was dissolved in Ethyl Acetate at a 20mg/ml concentration. Then the solution was added dropwise to 1ml of DI water with 2% PVA (W/V). The solution was sonicated using a probe sonicator (500W, Ultrasonic Processor GEX 500) with a 3 mm diameter microtip probe 3 times for 30 s at 40% amplitude with vortexing between intervals. The emulsion was added to 4ml of DI water containing 0.25% PVA (W/V) and the organic solvent was evaporated using a rotary evaporator (Buchi, Flawil, Switzerland) rotating at 120 rpm at room temperature for 15 minutes until all solvent was removed. The PLGA NPs were centrifuged and washed in DI water to remove the PVA and unencapsulated drug at 14000 rpm for 10 min at 4 °C 3 times. Then, the NPs were used fresh or frozen at –80 °C and lyophilized overnight (0.01 mbar at –87 °C).

Bare PLGA NPs loaded with water-soluble agents (AZT, BSA, Ovalbumin, or EGFP) were fabricated using the double emulsion/solvent evaporation method. Briefly, 1mg of drug/protein or 0.1mg of plasmid DNA in DI water was added to PLGA in ethyl acetate, sonicated for 10 s 3 times at 40% amplitude. Then, the procedure was similar to the empty bare PLGA synthesis as described above.

PLGA-liposomes and PLGA-ICMVs (empty or with ETR) were synthesized using the single emulsion/solvent evaporation method as well. A mixture of 1/1 molar ratio of DOPC and MPB lipids (total weight of 2.27mg) were dried from a chloroform solvent using a N₂ gas stream to form a lipid layer on a glass vial. The lipid films were dried from the chloroform residues overnight in a vacuumed dessicator. The following day, the lipids were hydrated in 1 ml of DI water and were vortexed every 10 minutes for 1 hr to remove the lipids from the vial walls. PLGA in ethyl acetate solution was added dropwise to the lipid hydration, vortexed, sonicated, added to 4ml of DI water, and evaporated as described above. At this point, for PLGA-liposomes, the NPs were purified using a PD-10 column (GE Healthcare, Little Chalfont, UK) following the manufacturer gravity protocol. We chose PD-10 column because centrifugation of the lipid containing NPs formed a sticky pellet that could not be resuspended in water.

For PLGA-ICMVs the synthesis was continued after evaporation of the organic solvent, 8 µl of 150mM DTT was added to the NPs and immediately after 0.016mmoles of CaCl₂ was added to the solution. The solution was incubated for 1 hr at 37°C in a 120 rpm orbital shaker. Then, the NPs were purified using a PD-10 column as described above.

For the PLGA-liposomes and PLGA-ICMVs with water-soluble agents we used the double emulsion/solvent evaporation procedure where the soluble agent is added to the PLGA organic solution before the organic solution is added to lipid hydration as described above.

2.3. Size and Zeta-potential

The hydrodynamic size, polydispersity (PDI), and zeta-potential in 10 mM NaCl of the PLGA particles were determined with a Malvern Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). Zeta-potential was evaluated using the Smoluchowski approximation.

2.4. Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM samples were prepared by the vitrification method where a drop of the solution was applied on a Lacey Carbon grid (TED Pella, Inc., Redding, CA), blotted, and plunged into liquid ethane. The samples were stored in liquid nitrogen until used. The samples were transferred to the microscope using a cryo transfer station and a Gatan 626 cryo-holder (Gatan, Inc., Pleasanton, CA). The samples maintained below -178°C during imaging. Imaging was done by the Tecnai G2 F20 transmission electron microscope (FEI Co., Hillsboro, OR) at 200kV with a field emission gun, in a low-dose mode to minimize electron-beam radiation damage. The micrographs were recorded by a 4K CCD camera (4k Eagle Camera, FEI).

2.5. Evaluation of Drug, Protein, and DNA Loading

To evaluate the encapsulation efficiency and drug loading of the agents ETR, AZT, BSA, OVA, and GFP plasmid the loaded NPs were frozen at -80°C and then lyophilized at 0.01 mBar and -87°C over night. Then, the lyophilized powder was measured using an analytical scale and afterward the NPs were dissolved in DMSO. To evaluate the loading of ETR and AZT we used UV-HPLC (Shimadzu), with a C18 column (Phenomenex, Torrance, CA). The samples were analyzed by the LCSolutions software. For ETR and AZT we used isocratic methods with flow rate of 1ml/min and the oven temperature maintained at 30°C . The buffers used for ETR were 65% acetonitrile (ACN) and 35% 10mM ammonium acetate in water. The injection volume was 10 μl , retention time was 5.7 min, and the detection wavelength was 234 nm. For AZT we used 72% water with 0.045% trifluoroacetic acid (TFA) and 28% CAN with 0.036% TFA. The retention time was 3.385 min and the wavelength was 265 nm. ETR and AZT were quantified using standard curves in DMSO. The loading of OVA and BSA was quantified by using the micro BCA assay (Pierce Biotechnology, Rockford, IL) according to the manufacturer protocol in 1% DMSO. Absorbance was recorded at 562 nm wavelength using a Tecan plate reader reader (Männedorf, Switzerland).

The GFP loaded NP powders (after lyophilization) were dissolved in chloroform and the GFP was extracted by using a chloroform-water extraction where water was added to the NPs in chloroform solution, mixed for 1 hr, and then the aqueous layer was removed by centrifugation at 9000 rpm for 10 min. This procedure was done 3 times to extract most of the DNA (extraction efficiency of 95% from a solution of empty NPs with DNA that was not encapsulated). The DNA was quantified using Quant-iT™ PicoGreen kit (Molecular Probes Inc., Eugene, OR) according to the manufacturer protocol with a Tecan plate reader in the fluorescence mode (excitation wavelength 480nm, emission wavelength 520nm).

Drug loading, encapsulation efficiency, and mass recovery were calculated with the following equations:

$$\text{Drug loading (DL) \%} = (\text{mass of drugs in NPs} / \text{total mass recovered from synthesis}) * 100$$

(1)

$$\text{Theoretical DL \%} = (\text{mass of drugs input} / \text{total mass input}) * 100 \quad (2)$$

$$\text{Encapsulation efficiency (EE) \%} = (\text{DL} / \text{theoretical DL}) * 100 \quad (3)$$

$$\text{Recovery \%} = (\text{total mass recovered from synthesis} / \text{total mass input}) * 100 \quad (4)$$

2.6. Cell Viability

TZM-bl cells were plated in a 96-well plate and incubated overnight. Then, empty NPs from each platform were added to cells at 0.5mg/ml, 1mg/ml, and 2mg/ml. These concentrations were chosen due to limitations of the PD-10 protocol that requires a defined volume of buffer to elute the NPs, thus maximum NP concentrations are established. NPs could not be further concentrated by centrifugation as the NP pellet is very sticky in the lipid-containing NPs and thus resuspension in solution is not feasible. The cells were incubated with the NPs for 48 hr and cell viability was evaluated using the CellTiter-Blue® assay (Promega, Fitchburg, WI) according to the manufacturer protocol. The fluorescence was recorded using a plate reader at emission/excitation wavelengths of 590/560 nm.

2.7 Dendritic Cell Derivation and Antigen-Specific CD4+ T Cell Proliferation Assay

Bone marrow dendritic cells (BMDCs) were derived from tibias and femurs of a C57BL/6 female mouse, 8–12 weeks old. Briefly, tibias and femurs were collected and the bone marrow was flushed out using a needle and syringe. The red blood cells collected were lysed and the remaining cells were cultured on petri-dishes in BMDC No. 10 media (2 mM L-glutamine, 10 mM HEPES, 1x non-essential amino acids, 1 mM sodium pyruvate, 55 µM 2-mercapthoethanol, 1% penicillin/streptomycin, and 10% fetal bovine serum) with 1 mg/mL GM-CSF (PeproTech, Rocky Hill, NJ). After 3, 5, and 7 days of incubation the cells were supplemented with new BMDC No. 10 media and GM-CSF. On Day 8, cells were detached, collected, and magnetically labeled with anti-CD11c microbeads (Miltenyi, Bergisch Gladbach, Germany) for collection of CD11c+ dendritic cells. CD11c+ DCs were isolated by positive selection using a magnetic column and used for further experimentation. CD11c+ DCs were incubated with EndoFit Ovalbumin (InvivoGen)-loaded PLGA nanoparticles, PLGA-liposomes, or PLGA ICMVs for a total of 5µg ovalbumin for 24 hours before co-culture with T cells.

CD4⁺ T cells were collected from the spleens of OT-II transgenic mice (Jackson Laboratories, Bar Harbor, ME). Briefly, spleens were collected, digested with DNase (40 µg/mL)/Collagenase D (1.5 mg/mL) solution, connective tissue was filtered out by a 70 µm strainer, and red blood cells were lysed. Cells were incubated with magnetically-labeled antibodies and CD4⁺ T cells were isolated by negative selection through a magnetic column (Milteni). CD4⁺ T cells were then labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE) (10 µM) (Thermo Fisher Scientific, Waltham, MA) for proliferation tracking. The CD4⁺ T cells from these transgenic mice have T cell receptors that recognize OVA_{323–339} and thus serve as an antigen-specific metric of T cell stimulation. T cells were co-cultured with particle-treated CD11c⁺ DCs at a ratio of DC:T cell 1:10. After 72 hours, cells were collected, stained with APC anti-mouse CD4 (BD Biosciences, San Jose, CA) and Live/Dead Fixable Violet Dead Cell Stain (Thermo Fisher Scientific), fixed with 1.6% paraformaldehyde, and analyzed by flow cytometry (BD LSR II, BD Biosciences). Results are reported as percentage of live, CD4⁺ T cells that underwent at least one round of proliferation determined by dilution of the CFSE signal using untreated CD4⁺ T cells as a control.

Concentration of cytokines IL-4 and IFN-γ that were secreted into the cell culture media after DC-T cell co-culture were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (PeproTech).

Statistical analyses were performed using GraphPad Prism 6 software using one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-test. Results are expressed as the mean ± standard deviation.

3. Results and Discussion

3.1 Synthesis of PLGA-lipid Hybrid Nanoparticles

We developed a reproducible synthesis method for PLGA-lipid and PLGA-ICMV core-shell NPs using an emulsion-solvent evaporation method (Figure 1). Our formulation was based according to previous reports of core-shell PLGA/lipids NPs synthesis at the ratio of 8/1. However, these NPs showed only one bilayer on the surface of the PLGA core instead of a multilayered structure that we expected (Figure S1, Supplementary Information). Therefore, we increased the lipid amount by 2-fold and changed the PLGA/lipids weight ratio to 8/2, where a multilamellar shell was able to be observed. Our synthesis was dependent on the type of biological agent that was encapsulated. For water-soluble agents (proteins, DNA, and hydrophilic drug) we used a double emulsion method (Figure 1A–G) whereas a single-emulsion method was used for hydrophobic drugs and empty NPs (Figure 1C–G). In the single-emulsion method, we dissolved PLGA in organic solvent and added it to the lipid hydration at a ratio of 1/2 organic/water phase, which was sonicated to form the oil-in-water emulsion. At this point, the lipids, due to their amphiphilic nature, acted as a surfactant to form the spherical core-shell NPs. The organic solvent was then evaporated. To synthesize PLGA-ICMVs, we added DTT after organic solvent evaporation to crosslink the maleimide groups present on the MPB lipid and immediately afterwards added CaCl₂ to screen the repulsion between the lipid bilayers, which facilitated the formation of multilamellar

bilayers on the surface of the PLGA, and therefore, helps in the crosslinking reaction. After the crosslinking reaction, we purified the NPs from unbound DTT using a PD-10 column.

All particles demonstrated similar size (150 nm) with low polydispersity index (PDI=0.1) (Table 1). However, zeta-potential differed between the platforms. The bare PLGA NPs were close to neutral charge whereas the PLGA-liposomes and PLGA-ICMV were more negatively charged. The zeta-potential of PLGA-ICMV was -46mV and the PLGA-liposomes was -42mV , in contrast to the zeta-potential of the bare PLGA NPs, which were around -2mV . The size and zeta-potential of the NPs showed that the incorporation of the lipids changed the resultant physico-chemical properties of PLGA particles. As expected, the zeta-potential of the bare PLGA NPs was close to neutral [15] but the addition of the lipid shell to the lipid-containing PLGA NPs changed the zeta-potential to be more negatively charged. While the synthesis of PLGA-liposomes resulted in particles with a negatively charged particles, the high standard deviation of the zeta potential suggests potential overall instability of PLGA-liposomes although the PDI remains low. A previous report showed that the zeta-potential of the PLGA-lipid hybrids changed according to the lipid ionic characteristics [4]. In our system, the lipids are anionic and zwitterionic and therefore our particles exhibit negative zeta-potential as expected.

3.2 Cryogenic Transmission Electron Microscopy of NPs

Nanostructure of the bare PLGA, PLGA-liposomes, and PLGA-ICMV NPs were imaged by cryo-TEM (Figure 2). Imaging showed that the bare PLGA NPs appeared to form a polymeric spherical structure (Figure 2A), and the PLGA-liposomes form a polymeric core and a lipid shell (Figure 2B, inset). This result agrees with the findings of Bershteyn et al. [10], in which only one lipid bilayer forms on the surface of the polymer when using a lipid mixture of zwitterionic and anionic lipids (DOPC and MPB in our system, respectively). For the PLGA-ICMV, we observed a polymeric core and multilamellar lipid bilayers on the surface (Figure 2C and Figure S2, Supporting Information). Based on analysis of cryo-TEM images, the majority of the nanoparticles (83.5%) were PLGA-ICMV, further supporting the successful synthesis of PLGA nanoparticles encapsulated in multilamellar lipid bilayers in addition to the observed change in particle zeta potential upon lipid-coating. These results demonstrate the formation of multilamellar layers despite that the system forms one lipid bilayer on the surface of the polymer in conditions without the crosslinking reaction. Thus, the crosslinking reaction induces the formation of multilamellar bilayers and stabilizes the formation of a polymer core with a shell of multilamellar lipid bilayers. Cryo-TEM also shows the morphological difference between the three types of the PLGA particles. Bare PLGA NPs consist of polymeric core only, PLGA-liposomes form one lipid layer on the surface of the polymeric core, and PLGA-ICMV form multilayered shell on the surface of the polymeric core. The morphological difference between the three types of particles shows that the synthesis of the PLGA-ICMV is successful and that we were able to crosslink between the lipid bilayers to create PLGA-ICMV.

3.3 Pharmaceutical Agent Encapsulation

To examine the loading capabilities of the different PLGA-lipid hybrid NPs, we incorporated different biological agents during formulation and compared loading efficiencies to bare

PLGA NPs (Table 2). First, we encapsulated the hydrophobic small molecule drug etravirine (ETR). For ETR-loaded PLGA-liposomes and PLGA-ICMVs, the size of the NPs increased when ETR was present and the zeta-potential became more negative. For PLGA-liposomes loaded with ETR, the PDI increased (0.2) and the standard deviation of the zeta-potential was high, indicating that inclusion of the hydrophobic drug destabilized the colloidal stability of the PLGA-liposome NPs. The encapsulation efficiency of ETR was 90% for the bare PLGA NPs, but for lipid-containing NPs the encapsulation efficiency dropped to 55% for the PLGA-liposomes and to 25% for the PLGA-ICMVs. We expect that this is probably because most of the drug is surface-associated with the PLGA core [16]. In contrast, the surface of PLGA-ICMVs and PLGA-liposomes is associated with the lipids and is less available for the drug to associate on it. Additionally, the PLGA surface is more hydrophobic than the surface of the lipid bilayers, and therefore the interaction of ETR with the surface of the lipid bilayers is less favorable. After this initial loss in hydrophobic drug association upon lipid coating of the particles, we expect further loss of drug encapsulation occurs in the lipid cross-linking step due to synthesis conditions (performed at 37 °C with orbital shaking at high rpm). Therefore, for hydrophobic drug delivery using the PLGA-lipid hybrids one should consider conjugating the drug on the surface of the NPs. For example, Zheng et al. [17] synthesized transferrin-conjugated lipid coated PLGA NPs loaded with aromatase inhibitor, which is a hydrophobic therapeutic drug for breast cancer. They tested several formulation parameters including lipid to PLGA ratio, drug to PLGA ratio, the lipid mixture, and aqueous to organic (solvent injection method) ratio. They demonstrated that their optimal formulation (PLGA-to-lipid 6/15 wt/wt and a drug-to-PLGA ratio of 0.015/1) had drug-loading efficiency of 36.3% and that their hybrid NPs were functional and able to enhance aromatase inhibition activity when targeted by transferrin conjugation on their surface.

For water-soluble agents, the lipid-containing nanoparticles showed better encapsulation efficiencies than bare PLGA NPs. PLGA-ICMVs were the best platform to encapsulate the hydrophilic drug AZT (42% EE), while bare PLGA (2% EE) and PLGA-liposomes (1.5% EE) showed poor AZT encapsulation. AZT did not alter the size or zeta-potential of bare PLGA NPs but did cause a slight increase in the size of PLGA-ICMVs and caused the PLGA-liposomes to have a more negative zeta-potential. The poor EE efficiency of the bare PLGA NPs was expected, as hydrophilic drugs tend to partition from the organic solvent droplets to the aqueous phase during the emulsification methods [20]. Fang et. al [21] also reported low EE of the hydrophilic drug salidroside by lipid-PLGA hybrids. Higher encapsulation efficiencies were only achieved only after incorporation of PLGA-PEG block copolymers. The superior EE of our PLGA-ICMVs system is likely due to the multilamellarity of the lipid shell, which provides additional hydrophilic regions within the nanoparticle where the hydrophilic drug can incorporate. Next, we sought to explore the incorporation of larger water-soluble molecules and we chose BSA as a model protein. Loading of the protein BSA caused an increase in the NP size for all platforms; however, when added to the PLGA-liposomes we observed large aggregation and particles that were larger than 1µm with very high PDI (0.44). BSA encapsulation efficiency was highest for PLGA-ICMVs (72%), intermediate for PLGA-liposomes (44%), and lowest for bare PLGA (35%). While BSA was able to be encapsulated in PLGA-liposomes, this is not suitable

nanoparticle delivery platform when synthesized as described here as it resulted in aggregation and the formation of micron-sized particles. As we observed a high standard deviation in the zeta potential of these particles even in the absence of therapeutic loading, the incorporation of BSA may further encourage their aggregation. However, Hu et al. [23] showed that tailored lipid-PLGA hybrids with PEG and cholesterol could increase the NP stability and the efficiency of the loading to 88–99%, depending on the amount of BSA loaded. It is likely that proteins destabilize our PLGA-liposome NPs because they do not contain PEG, which is known to form spaces between the NPs due to steric effects and prevent aggregation.

PLGA-ICMV's were the most effective in encapsulation of plasmid DNA, with 10% EE as compared to ~1% for both bare PLGA NPs and PLGA-liposomes. Inclusion of plasmid DNA did not have a big effect on the size nor zeta-potential of PLGA-ICMV's and bare PLGA NPs but caused the zeta-potential of PLGA-liposomes to become more negative (–77mV) compared to the empty PLGA-liposomes (–15mV). Bose *et al.* [22] characterized the ability of PLGA-lipid hybrids to deliver plasmid DNA. They used the cationic lipid DOTAP and showed that as the DOTAP composition in the lipid shell increased, the DNA incorporation was better and transfection efficiency increased. We expect that our system could be adjusted for DNA delivery by incorporation of cationic lipid such as DOTAP. Because our system consists only of anionic and zwitterionic lipids, the interactions between the DNA and the lipid are weak and therefore the encapsulation efficiency is low. Our results showed that the encapsulation efficiencies of the water-soluble agents AZT, BSA, and plasmid DNA by PLGA-ICMV's NPs (44%, 72%, and 10%, respectively) were better than with the bare PLGA NPs and PLGA-liposomes. While none of the platforms encapsulated the EGFP plasmid efficiently, the best platform was the PLGA-ICMV's. We hypothesize that the water-soluble agents prefer to interact with nanoformulations that allow more water association, such as the PLGA-ICMV's that consist of water gaps between the lipid lamellas.

3.4 Nanoparticle Cytotoxicity

We used the TZM-bl cell line to evaluate cell viability to further understand the biological potential of the different PLGA NP platforms (Figure 3). Cells that were treated with the PLGA-ICMV's showed the best viability for all three concentrations (88–86% viability). Cells that were treated with bare PLGA NPs also showed good viability, especially at the lowest concentration (88% viability), which was similar to the PLGA-ICMV's. At the highest concentration of bare PLGA NPs, the viability of the cells decreased to 70%. The PLGA-liposomes were the most toxic to the cells, especially at the highest concentration (2 mg/ml), where the cell viability was only 6%. We expect that the low viability is due to aggregation that occurred when the PLGA-liposomes were introduced to the cell media, which was observed visually. The aggregation of the PLGA-liposome NPs in the cell media further demonstrate low colloidal stability of these NPs, which causes changes in the morphological and physical properties that affect the interactions between the cells and the NPs and therefore, may induce cell toxicity. However, at lower concentrations of PLGA-liposomes (1 mg/ml and 0.5 mg/ml), the cell viability increased to 46% and 68% respectively. We also did not visually observe the formation of big aggregates at these concentrations. Yu *et al.* [24] reported low toxicity of a similar PLGA-lipid hybrid system. Their PLGA-liposome system

contained DSPE-PEG lipid that contributed to the NP stability where the PEG chains may help to prevent excessive aggregation. In our system, the PLGA-liposomes did not exhibit such stability in contrast to the PLGA-ICMVs that preserved their colloidal stability in the cell culture medium. Thus, we conclude that the PLGA-ICMVs platform is an attractive delivery platform for many biological agents because of its low toxicity and superior ability to encapsulate different kinds of biological agents.

3.5 Antigen-specific CD4+ T cell Activation

Finally, we evaluated the functionality of the different PLGA-based particulate platforms and determined the ability to deliver a protein antigen and initiate adaptive immune responses. CD11c+ bone marrow-derived dendritic cells (DCs), professional antigen presenting cells, were treated with each OVA-loaded PLGA particle platform. All of our nanocarriers were loaded with equivalent mass of OVA (5µg) and the encapsulation efficiencies of OVA in PLGA NPs, PLGA-liposomes (PLGA LPs), and PLGA ICMVs were 34.6%, 25.7%, and 58.0%, respectively. All OVA-loaded particle platforms induced CD4+ T cell proliferation (Figure 4A) when NP-treated DCs were co-cultured with antigen-specific T cells and increased the percentage of CD4+ T cells that underwent at least one round of proliferation compared to particle control groups that did not contain OVA (Figure 4C). Among the particle groups, OVA delivery in PLGA-ICMVs to DCs resulted in the highest CD4+ T cell proliferation, which was the only particle group statistically equal to the soluble OVA positive control.

We next investigated the production of IFN-γ and IL-4 cytokines after naïve CD4+ T cells were incubated with OVA-loaded particle-treated dendritic cells. Upon activation, T cells may differentiate into a certain lineage of helper cells with distinct cytokine profiles and functions [25]. We detected IFN-γ, the signature cytokine of T helper-1 (T_H1) cells but no IL-4, a signature cytokine of T_H2 cells, in culture supernatants of all OVA-loaded particle groups (Figure 4B). These results indicate that protein-loaded particles induced T_H1 polarization of the CD4+ T cells. IFN-γ was not detected in the vehicle controls that were not loaded with OVA, demonstrating antigen-specific production of inflammatory cytokines. All OVA-loaded particles resulted in a statistically significant increase in IFN-γ cytokine secretion compared to the soluble OVA treatment (no NP carrier), indicating a functional role of the particles to induce stronger T cell responses than unformulated antigens. IFN-γ is a critical immune cytokine that has anti-viral and anti-parasitic activity, can activate macrophages to kill tumor cells, and induce the proliferation of activated B cells. The increased levels of IFN-γ in the particle groups also points to the ability of PLGA-based nanocarriers to act as an adjuvant with low cytotoxicity profiles in vaccine delivery, with lipid-coated PLGA particles (PLGA-ICMVs and PLGA-liposomes) as the most promising platforms for this application. Enhanced T cell responses have been observed for both ICMVs and PLGA nanoparticle vaccine systems. Moon *et al.* found that protein-based antigens delivered to dendritic cells in ICMVs could be better cross-presented to CD8+ T cells, resulting in enhanced CD8+ T cell proliferation compared to soluble antigen delivery [18]. Similarly, antigen-loaded PLGA nanoparticles have been shown to enhance both CD4+ and CD8+ T cell proliferation when delivered to antigen presenting cells and these systems were also shown to induce the production of IFN-γ by T cells [26, 27].

4. Conclusions

Our study describes the synthesis of novel polymer-lipid hybrid nanoparticles, PLGA-ICMVs, that can be used to encapsulate a variety of therapeutic agents. This PLGA-ICMVs platform is an attractive platform for the stable, high encapsulation of water-soluble biological therapeutics and can mediate strong adaptive immune response when delivering an antigen payload. By comparing three PLGA-based nanoparticle delivery systems, we demonstrate that the formulation platform should be chosen according to the therapeutic that is to be encapsulated, with bare PLGA particles most favorable for hydrophobic small molecules and PLGA-ICMVs favorable for hydrophilic small molecules and water-soluble biologics such as proteins and DNA plasmids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Synthesis of PLGA-liposomes and PLGA-ICMVs. Step B is done only when adding a water-soluble biological agent such as hydrophilic drugs, proteins or nucleic acids. Hydrophobic drugs are added directly to the PLGA organic phase in A. Steps A-E) form PLGA-liposomes, and further synthesis forms the PLGA-ICMVs. A) PLGA is dissolved in organic phase (ethyl acetate). B) water soluble biological agent is added to the PLGA organic phase and emulsify by vortexing and sonication. C-D) The PLGA organic phase or emulsion is added to a liposome aqueous phase. E) The mixture is emulsified by sonication. In this stage, the PLGA-liposomes NPs are formed. F) crosslinking reaction with DTT and addition of ions to form the multilamellar crosslinked bilayer shells. G) Purification with PD-10 column or dialysis to remove excess of DTT and/or unbound biological agents.

Figure 2:
Cryo-TEM images of A) bare PLGA NPs, B) PLGA-liposomes, and C) PLGA-ICMVs. B) The black arrows indicate particles with a PLGA core and lipid shell. The inset shows a nanoparticle with a polymeric core and a lipid shell on its surface. C) The white arrow indicates a PLGA core with few lipid bilayers on the surface. White asterisks indicate the dense polymeric core of the NPs.

Figure 3:
TZM-bl cell cytotoxicity assay with cell titer blue. All values represented as mean $\pm$ S.D from triplicates.

Figure 4:

Evaluation of NP platforms as a vaccine delivery system. A) Flow cytometry gating strategy for quantification of proliferating, live, CD4+ T cells. B) OVA-specific CD4+ T-cell proliferation after BMDC treatment with OVA-loaded particles. C) IFN- γ levels in the T-cell culture supernatants. Particle-treated groups were compared against the soluble ovalbumin control (no vehicle, +OVA) using one-way analysis of variance and Tukey's multiple comparison post-test. Statistical significance was defined as $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$).

Table 1:

Properties of blank NPs in 10mM NaCl, pH~6. Mean and $\pm$ S.D calculated from n>3.

	Size (nm)	PDI	ζ -potential (mV)
Bare PLGA	157.0	0.1	-2.2 $\pm$ 3.8
PLGA-liposomes	144.1	0.1	-42.2 $\pm$ 38.5
PLGA-ICMVs	136.3	0.1	-46.5 $\pm$ 9.2

Table 2:

Properties of NPs loaded with different biomolecules. Mean and $\pm$ S.D calculated from n=3, except from ETR n=2.

		Size (nm)	PDI	Zeta (mV)	Theoretical DL %	DL%	EE%	Recovery%
Etravirine (ETR) loading	Bare	151.0 $\pm$ 0.3	0.03	-1.7 $\pm$ 42.4	4.77	4.3 $\pm$ 0.4	90.3 $\pm$ 1.3	4.4 $\pm$ 13.2
	PLGA							
	PLGA- liposomes	125.3 $\pm$ 1.4	0.20	-41.7 $\pm$ 39.1	3.92	3.2 $\pm$ 0.6	54.8 $\pm$ 17.8	20.6 $\pm$ 6.7
	PLGA- ICMV _s	193.2 $\pm$ 2.5	0.10	-56.6 $\pm$ 7.8	3.86	0.99 $\pm$ 0.3	25.5 $\pm$ 7.3	46.3 $\pm$ 11.0
Azidothymi dine (AZT) loading	Bare	136.7 $\pm$ 4.9	0.06	-1.1 $\pm$ 7.4	9.09	0.19 $\pm$ 0.06	2.18 $\pm$ 0.7	21.0 $\pm$ 5.7
	PLGA							
	PLGA- liposomes	124.2 $\pm$ 8.3	0.13	-36.4 $\pm$ 10.4	7.53	0.11 $\pm$ 0.1	1.47 $\pm$ 1.2	17.6 $\pm$ 7.9
	PLGA- ICMV _s	170.3 $\pm$ 82.8	0.08	-36.9 $\pm$ 6.1	6.56	2.8 $\pm$ 0.1	42.4 $\pm$ 2.0	50.3 $\pm$ 21.2
Bovine Serum Albumin loading	Bare	169.0 $\pm$ 0.2	0.17	-5.8 $\pm$ 7.6	9.09	3.2 $\pm$ 1.6	35.5 $\pm$ 18.1	29.0 $\pm$ 13.7
	PLGA							
	PLGA- liposomes	> 1 μ m	0.44	-49.4 $\pm$ 7.0	7.53	3.3 $\pm$ 0.8	44.2 $\pm$ 11.2	34.2 $\pm$ 10.4
	PLGA- ICMV _s	237.3 $\pm$ 143.0	0.10	-43.6 $\pm$ 7.2	6.56	4.8 $\pm$ 2.1	72.0 $\pm$ 31.7	56.9 $\pm$ 30.8
GFP plasmid loading	Bare	143.7 $\pm$ 6.3	0.04	-2.4 $\pm$ 0.8	0.99	0.01 $\pm$ 0.01	1.23 $\pm$ 1.3	52.5 $\pm$ 33.6
	PLGA							
	PLGA- liposomes	126.3 $\pm$ 23.8	0.08	-77.4 $\pm$ 0.6	0.81	0.01 $\pm$ 0.01	1.36 $\pm$ 1.3	73.8 $\pm$ 21.0
	PLGA- ICMV _s	115.7 $\pm$ 11.3	0.09	-42.5 $\pm$ 3.3	0.79	0.08 $\pm$ 0.05	10.0 $\pm$ 6.8	63.9 $\pm$ 14.9