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TIPS Pentacene Loaded PEO-PDLLA Core-Shell Nanoparticles have Similar Cellular Uptake Dynamics in M1 and M2 Macrophages and In Corresponding *In Vivo* Microenvironments

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Abstract

Nanoparticle based drug delivery platforms have the potential to transform disease treatment paradigms and therapeutic strategies, especially in the context of pulmonary medicine. Once administered, nanoparticles disperse throughout the lung and many are phagocytosed by macrophages. However, there is a paucity of knowledge regarding cellular up-take dynamics of nanoparticles due largely to macrophage heterogeneity. To address this issue, we sought to better define nanoparticle up-take using polarized M1 and M2 macrophages and novel TIPS-pentacene loaded PEO-PDLLA nanoparticles. Our data reveals that primary macrophages polarized to either M1 or M2 phenotypes have similar levels of nanoparticle phagocytosis. Similarly, M1 and M2 polarized macrophages isolated from the lungs of mice following either acute (Th1) or allergic (Th2) airway inflammation also demonstrated equivalent levels of nanoparticle up-take. Together, these studies provide critical benchmark information pertaining to cellular uptake dynamics and biodistribution of nanoparticles in the context of clinically relevant inflammatory microenvironments.

GRAPHICAL ABSTRACT

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Nanoparticles are promising drug delivery and bioimaging platforms. However, the cellular uptake dynamics of nanoparticles are not fully understood, especially in the case of macrophages that can differentiate into different M1 or M2 polarization states to tightly regulate inflammation. In this study, the cellular uptake dynamics of nanoparticles were investigated in polarized macrophages and in the context of either acute or allergic lung inflammation. The overall findings of this paper demonstrate that M1 and M2 macrophages display similar cellular uptake dynamics both *ex vivo* and *in vivo* under corresponding Th1 and Th2 modulated microenvironments.

Keywords

asthma; LPS; nanoparticle; pulmonary drug delivery; inflammation

BACKGROUND

A diverse range of inflammatory airway diseases are associated with dysregulated Th1 or Th2 microenvironments that could potentially be ameliorated through nanoparticle-mediated drug delivery or benefit from nanoparticle-assisted bioimaging applications. However, efforts to fully utilize the potential of nanoparticle based therapeutics in human disease applications is currently limited and requires a significant, comprehensive understanding of host-nanoparticle interactions. A highly relevant obstacle for *in vivo* nanoparticle use in mucosal tissues is rapid phagocytosis and clearance by phagocytic cells¹⁻³. This phagocytosis can be highly variable among individual patients, which can result in unpredictable pharmacokinetics of nanoformulations⁴. In the lungs, phagocytic cell

composition and function is significantly impacted by the inflammatory microenvironment, which is regulated in large part by the balance of Th1 and Th2 associated cytokines. Under Th1 conditions, macrophages typically polarize to a pro-inflammatory M1 state, which is the predominant phenotype observed in the lung during pathogen infection and in the context of chronic inflammatory diseases, such as chronic obstructive pulmonary disease (COPD)^{5,7}. Under Th2 conditions, macrophage polarization is skewed towards an M2 phenotype. In the lung, the contribution of M2 macrophages in disease pathogenesis is less defined compared to M1 macrophages. However, M2 macrophages have been shown to produce a range of both pro- and anti-inflammatory mediators, depending on specific microenvironmental conditions^{8,9}. A diverse spectrum of human lung diseases are associated with Th2 mediated immunity, including asthma and lung cancer¹⁰.

The presence of M1 and M2 macrophage populations in the lungs, coupled with the distinct inflammatory microenvironments during Th1 and Th2 mediated airway inflammation, represent a significant challenge to understanding nanoparticle up-take dynamics and utility during disease. In general, prior studies have revealed a significant increase in nanoparticle phagocytosis and clearance under Th2 conditions^{3,11}. This appears to be associated with increased M2 macrophage polarization and recruitment^{3,11}. This does not appear to be a universal finding, as other studies have reported increased phagocytosis by M1 macrophages^{12–14}. It is highly likely that these discrepant findings are significantly influenced by nanoparticle composition, size, chemical modifications, cell polarization method, and mouse strain differences³.

The majority of prior studies evaluating M1 and M2 macrophage phagocytosis and uptake have focused on *ex vivo* observations with limited *in vivo* studies. However, there have been a few studies which have shown successful therapeutic delivery and efficacy *in vivo* regarding macrophages and dendritic cells in inflammatory diseases and cancer^{15–18}. Specifically, the studies involving macrophages showed cellular uptake both *in vitro* and *in vivo*. Furthermore, the nanoparticles used were “loaded” with therapeutics to investigate the effect on disease models^{15–17}. Together, these studies indicate the usefulness of nanoparticles as drug delivery vehicles to macrophages, which presented significant nanoparticle uptake. However, the relative lack of *in vivo* work is due, in part, to the lack of an effective model system with sufficient numbers of polarized macrophages to effectively study uptake. Thus, it is difficult to extrapolate the findings from these prior *in vitro* studies into clinically and physiologically relevant models of macrophage uptake dynamics in the context of human disease. To address this issue, in the current study, we characterized novel poly(ethylene oxide-b-D,L-lactide) (PEO-PDLLA) diblock core-shell nanoparticles loaded with the fluorescent dye TIPS pentacene and investigated the difference in cellular uptake of these nanoparticles by primary M1 and M2 macrophages both *ex vivo* and *in vivo* in Th1 and Th2 microenvironments in the lung.

METHODS

Nanoparticle Fabrication

Nanoparticles comprised of PDLLA cores with PEO shells were fabricated using Flash Nanoprecipitation^{19,20}. Briefly, PEO-b-PDLLA diblock copolymer and TIPS pentacene

were dissolved separately in uninhibited THF by vortex mixing followed by 30 minutes sonication for the polymer and 15 minutes for the fluorophore. The two solutions were then combined to have final concentrations of 33 mg/mL for the polymer, and 0.33 or 0.66 mg/mL for the fluorophore depending on the targeted loading. The mixture was then syringe filtered (0.2 μ m Nylon) and sonicated for an additional 15 minutes. Using a 5 mL glass syringe, the organic solution was fed into a four jet multi-inlet vortex mixer (MIVM) at a flow rate of 9.99 mL/min using a syringe pump (New Era Pump Systems). Deionized (DI) water was fed simultaneously by three separate 60 mL syringes at flow rates of 33.3 mL/min using a manually programmed pump (Harvard apparatus PHD 4000) so that the THF/water volumetric ratio in the stream exiting the mixer was 1/10. The dimensions of the MIVM were similar to a design previously described²⁰, under the flow conditions used, the Reynolds number in the mixer was approximately 13,500. The exiting nanoparticle suspension was collected and dialyzed in 4L of water for 24 hours. The sample was then lyophilized for 6 days at conditions <0.09 mBar and $\sim$ -50°C. The freeze-dried powder was transferred to sealed glass vials, and wrapped in foil for storage at 4°C.

Nanoparticle Characterization

The hydrodynamic diameter and zeta potential were measured using a Malvern Zetasizer Nano ZS instrument. Freeze-dried nanoparticles were re-dispersed in DI water at an approximate concentration of 0.1 mg/mL. The suspension was vortex mixed and sonicated for 30 minutes. Dynamic light scattering (DLS) measurements were taken at 37°C with an equilibration time of 600 seconds in disposable polystyrene cuvettes. Zeta potentials were measured by re-dispersing freeze-dried nanoparticles in 0.01 $\times$ PBS at approximately 0.1 mg/mL. The solution was vortex mixed and sonicated for 30 minutes again before being loaded into a folded polystyrene capillary cell for measurement at 37°C with an equilibrium time of 600 seconds. The absorbance and fluorescence measurements were taken for TIPS pentacene dissolved in THF. The absorbance was measured using an Evolution 300 UV-Visible Spectrophotometer and scanned from 400–700 nm. A Cary Eclipse Fluorescence Spectrophotometer (Agilent) was used to obtain the fluorescence spectrum from 600–800 nm. Both spectra were normalized to the peak intensity. The encapsulation efficiency was calculated using the absorbance of the TIPS pentacene in the particles at 594 nm and finding the concentration of TIPS pentacene in a known concentration of nanoparticles in THF. The method is further described in the supplementary information.

Experimental Animals

All mouse studies were approved by the IACUC at Virginia Tech and in accordance with the *Federal NIH Guide for the Care and Use of Laboratory Animals*. All studies utilized wild type C57Bl/6J mice.

Macrophage Polarization and Immunogenicity Assessments

Bone marrow derived macrophages (BMDMs) were harvested from mice using standard protocols²⁹. Briefly, BMDMs were grown for 6 days in DMEM supplemented with 10% fetal bovine serum, L-Glutamine, 1X penicillin/streptomycin, and 20% L929-conditioned medium. Following the 6 day culture, BMDMs were polarized to M1 or M2 macrophages by re-plating in L929-free media, containing either IFN γ (10 ng/mL) or IL-4 (10 ng/mL) to

polarize the cells to an M1 phenotype or M2 phenotype, respectively. Polarization was confirmed via rtPCR for gene markers associated with M1 (*Tnf* and *Ifng*) and M2 (*Ym1*, *Fizz* and *Arg1*) macrophages. LPS (50 ng/mL) was used as a positive control. Cells were treated with PEO-PDLLA diblock nanoparticles (100 µg/mL) for 2, 4, and 24 hours. At these time points, supernatants were harvested and cytokine levels were measured using ELISA. Lactate dehydrogenase (LDH) activity in the supernatant was used to quantify PEO-PDLLA nanoparticle cytotoxicity.

Visualization and Quantification of Nanoparticle Phagocytosis

Nanoparticle phagocytosis and cellular up-take was quantified and imaged using an Amnis® ImageStream imaging flow cytometer system. Auto-fluorescence was used to visualize cells. Cell viability was assessed using Hoechst (10 µg/mL) dye as a pre-treatment prior to flow cytometry evaluation. The incorporation of the TIPS pentacene allowed for the intercellular visualization and quantification of the nanoparticles. At least 10,000 cells were evaluated for each condition.

Induction and Evaluation of Nanoparticle Distribution in Th1 and Th2 Microenvironments in the Lung

To model acute lung inflammation associated with Th1 mediated inflammation, mice were treated with bacterial LPS. Intratracheal (i.t.) administration of LPS (1 µg/g in 50 µl of PBS) was given following previously described protocols²¹. To model allergic lung inflammation associated with Th2 mediated inflammation, mice were given intraperitoneal (i.p.) injections of OVA (20 µg in aluminum hydroxide) on days -21 and -7, in order to sensitize the mice. On days 21 through 25, mice were given OVA (1% in PBS) daily via intranasal (i.n.) administration following previously described methods²²⁻²⁵. In both models, nanoparticles were administered by i.t. administration 24 hours following the last lung exposure of either LPS or OVA. Twenty-four hours after nanoparticle administration, mice were euthanized and tissues were harvested as previously described¹. To quantify *in vivo* cellular up-take of nanoparticles, BALF cells were evaluated using the imaging flow cytometer. Expanded harvest procedures are available in the Supplemental Procedures.

Statistical Analysis

Single data point comparisons were evaluated by Student's two-tailed t-test. Multiple comparisons were evaluated for significance using ANOVA followed by either Tukeys or Newman-Keuls post-test. All data are presented as mean ± SD with p-values < 0.05 considered significant. Data shown are representative of at least 3 independent studies.

RESULTS

PEO-PDLLA Core-Shell Nanoparticles and TIPS Pentacene Have Useful Properties for Biomedical Applications

To identify the optimal parameters for the *in vitro* and *in vivo* detection of the PEO-PDLLA nanoparticles, we determined the UV-Vis absorbance and fluorescence emission spectra for TIPS pentacene in tetrahydrofuran (THF) (Figure 1A). TIPS absorbs in the orange with a prominent peak at 594 nm and emits strongly in the red at 650 nm. Based on these optical

properties, we determined that the optimal wavelength for detection by flow cytometry was 650 nm (Figure 1A). Using these settings, we were able to both quantify and visualize nanoparticle uptake without interfering with other assessed parameters and cellular auto-fluorescence.

Given that the size and charge of nanoparticles plays a significant role in uptake and drug loading efficacy^{2,26,27}, we characterized the size distribution of the PEO-PDLLA nanoparticle solution using Dynamic Light Scattering (DLS) (Figure 1B). Prior studies have shown that loading nanoparticles with molecules, such as fluorescent dyes, can significantly influence their size²⁸. Thus, we wanted to determine the effects of TIPS pentacene loading on the characteristics of our nanoparticles. To evaluate this, we synthesized nanoparticles with two different target loadings of TIPS pentacene: 1.0 and 2.0 weight percent (wt%) with encapsulation efficiencies at 98%. Increasing the target wt% of TIPS pentacene from 1.0 to 2.0, while keeping the PEO-PDLLA diblock concentration fixed, resulted in the intensity-average hydrodynamic diameter of the nanoparticles increasing from 90 nm to 115 nm (Figure 1B). The zeta potential of the PEO-PDLLA nanoparticles was ~-14 mV and the PDI <0.28. Neither parameter was sensitive to the fluorophore loading. Moreover, the PDI did not vary significantly with the TIPS pentacene loading (Figure 1C). Based on this insensitivity of the ZP and PDI to the target TIPS pentacene loading, we utilized particles with 1.01 wt% loading for the remainder of the study.

M1 and M2 Polarized Macrophages Display Similar Cellular Uptake of PEO-PDLLA Nanoparticles Ex Vivo

To examine the effect of macrophage polarization on PEO-PDLLA nanoparticle up-take, BMDMs were harvested from C57Bl/6J mice and polarized to either M1 or M2 phenotypes 24 hours prior to nanoparticle exposure²⁹. Polarization was confirmed using rtPCR with M1 cells expressing higher levels of *Tnf* and *Ifng* compared to the M2 phenotype, while the M2 polarized cells expressed higher levels of genes typically associated with the M2 phenotype, including *Ym1*, *Fizz*, and *Arg1*³⁰ (Figure 2A). Nanoparticle uptake was assessed via imaging flow cytometry and the percentage of cells positive for nanoparticles was quantified (Figure 2B–C). At least 10,000 cells were evaluated and imaged for each condition. Using these methods, we did not observe a significant difference in the percentage of cells positive for the TIPS pentacene-loaded PEO-PDLLA nanoparticles between M1 or M2 polarized macrophages either 2, 4, 8, 24, or 48 hours after treatment (Figure 2B–C). Within the first 4 hours post-nanoparticle exposure, approximately 20–40% of the macrophages had phagocytosed the nanoparticles and by 24 hours approximately 80% of the macrophages contained nanoparticles regardless of M1 or M2 polarization (Figure 2B–E).

PEO-PDLLA Nanoparticles are Non-Immunogenic and Non-Cytotoxic in M1 and M2 Macrophages

We next sought to assess immunogenicity and cytotoxicity of the TIPS pentacene-loaded PEO-PDLLA nanoparticles. Primary BMDMs were isolated, polarized, verified, and exposed to nanoparticles. Supernatants were collected and evaluated for the concentration of pro-inflammatory cytokines, including IL-6 and TNF, via ELISA (Figure 3). Here, we did not observe a significant increase in either IL-6 or TNF from macrophages exposed to

nanoparticles compared to mock treated cells, regardless of polarization state (Figure 3A–D). In addition to evaluating the nanoparticle immunogenicity, we also evaluated cytotoxicity using an LDH activity (Figure 3E–F). We did not observe a significant increase in LDH activity in either M1 or M2 polarized macrophages following nanoparticle exposure compared to the mock treated cells (Figure 3E–F).

TIPS Pentacene-Loaded PEO-PDLLA Nanoparticles Have Similar Uptake Dynamics in the Lungs during Acute and Allergic Airway Inflammation

To determine *in vivo* nanoparticle up-take dynamics by M1 macrophages and biodistribution during Th1 mediated acute lung inflammation, mice were treated with LPS via i.t.administration²¹. Nanoparticles were administered 24 hours following LPS exposure (Figure 4A). To evaluate immune cell recruitment to the lungs, we characterized the cellular components of the bronchoalveolar lavage fluid (BALF). As anticipated, LPS administration resulted in a significant increase in neutrophil recruitment to the lungs, with minimal effects on macrophage and lymphocyte populations (Figure 4B). Lung histopathology assessments confirmed the BALF findings and revealed robust lung inflammation and neutrophilia (Figure 4C–D). Consistent with our findings in the *ex vivo* models, administration of the TIPS pentacene-loaded PEO-PDLLA nanoparticles did not induce a significant immune response in the naïve mice (Figure 4B–D). Nanoparticle administration in the context of LPS induced acute lung inflammation did not influence macrophage or lymphocyte populations (Figure 4B–D). However, we did routinely observe a moderate decrease in BALF neutrophils following LPS exposure in mice treated with nanoparticles compared to the LPS exposed mice without nanoparticles (Figure 4B). To further evaluate lung inflammation, we also assessed local levels of pro-inflammatory cytokines associated with Th1 mediated inflammation in the BALF. LPS administration resulted in significant increases in pro-inflammatory Th1 associated cytokines, such as IL-6, regardless of nanoparticle administration (Figure 4E). Likewise, consistent with our findings that the nanoparticles were non-immunogenic, IL-6 was below the level of detection in the lungs from mice in the nanoparticle only treatment group (Figure 4E).

Based on these findings, we next sought to evaluate the cellular up-take of nanoparticles in the context of Th1 mediated acute airway inflammation. Following LPS and nanoparticle exposure, cells were harvested from BALF and prepared for imagingflow cytometry assessments. The TIPS pentacene-loaded PEO-PDLLA nanoparticles were detected in cells isolated from both PBS exposed mice treated with nanoparticles and in cells isolated from LPS exposed animals treated with nanoparticles (Figure 5A–B). In the PBS group, we observed that the majority of BALF cells assessed contained nanoparticles and was not significantly different from the LPS/NP group (Figure 5A).

In addition to Th1 mediated acute lung inflammation, we also sought to evaluate nanoparticle up-take dynamics in the context of Th2 mediated allergic lung inflammation. OVA administration resulted in significantly increased lymphocyte and eosinophil recruitment compared to the PBS groups (Figure 6B). However, nanoparticle administration did not significantly affect the BALF cellularity. Histopathology assessments confirmed that OVA administration resulted in the development of allergic lung inflammation and no

significant differences were observed between disease pathogenesis following nanoparticle administration (Figure 6C–D). Interestingly, mice that were exposed to OVA and treated with nanoparticles showed a higher HAI score compared to mice that received OVA only (Figure 6D). Perhaps during Th2 mediated disease, PEO-PDLLA nanoparticles may slightly promote inflammation seen in histopathological analysis. Consistent with the established Th2 microenvironment, OVA mediated allergic airway inflammation is associated with reduced levels of Th1 associated mediators, such as IL-6. Thus, we evaluated IL-6 in the BALF and counter to the levels observed following LPS exposure, the IL-6 levels following OVA administration were highly suppressed and not significantly increased over the PBS groups (Figure 6E). However, there was a slight significant increase in BALF IL-6 in mice exposed to OVA and treated with nanoparticles. In combination with the HAI data mentioned earlier, these data confirm that our PEO-PDLLA nanoparticles may slightly promote inflammatory signaling during Th2-mediated disease.

Similar to the findings reported for nanoparticle up-take under Th1 mediated conditions, we also observed high numbers of cells containing the TIPS pentacene-loaded PEO-PDLLA nanoparticles in the context of Th2 mediated allergic airway inflammation (Figure 7A–B). Nanoparticles were identified in cell populations from all of the treated animals (Figure 7A). No significant difference was observed between the number of cells containing nanoparticles from mice that were exposed to PBS/nanoparticles (8.20×10^4) compared to the mice that were exposed to OVA/nanoparticles (5.55×10^4) (Figure 7A). Overall, these uptake data are highly similar to the uptake data from the Th1-mediated microenvironment and show that there are a high number of cells that phagocytose nanoparticle in the lung in both naïve and disease conditions.

No Significant Difference in M1 and M2 Macrophage Up-Take In Vivo

While the quantification of the total number of nanoparticle containing cells yielded insightful data, these assessments evaluated all of the cells present in the BALF samples (Figures 5 and 7). Thus, to assess the up-take of the TIPS pentacene-loaded PEO-PDLLA nanoparticles by M1 and M2 macrophages *in vivo*, we utilized imaging flow cytometry. The percent nanoparticle up-take was calculated based on sorted macrophage populations from the BALF collected following LPS and OVA exposure (Figure 8). Using the flow cytometry data, macrophages were gated and those containing nanoparticles were quantified. Using this approach, no significant differences were found between the percent up-take of macrophages evaluated following LPS exposure (M1) compared to macrophages evaluated following OVA exposure (M2) (Figure 8). Our data indicate that, under our conditions, between 6.83 – 9.55% of the macrophages isolated from the lungs of inflamed mice contained TIPS pentacene-loaded PEO-PDLLA nanoparticles regardless of the underlying Th1/Th2 microenvironment.

DISCUSSION

As the use of nanoparticle-based drug delivery platforms and bioimaging agents become more mainstream, it will be essential to fully characterize nanoparticle formulations and their respective interactions with the host immune system. Due to the novelty of the

nanoparticles utilized in this study, we first sought to determine the major characteristics that could impact biodistribution and up-take by phagocytic cells. Previous reports have shown that nanoparticles between 10–100 nm are ideal for drug delivery³³. The rationale for this size range is due to prior studies using systemic nanoparticle delivery that indicated smaller particles were easily cleared from the circulation via the kidneys, while larger particles accumulate in sinusoids and were eventually cleared via the mononuclear phagocyte system (MPS)². Similar findings have also been reported for lung deposition³⁴. In addition to size, nanoparticle surface charge has also been shown to play an important role in cellular uptake and drug delivery^{35–37}. For example, negatively charged nanoparticles with zeta potentials ranging from ~0 to –20 mV are typically favored for therapeutic delivery. Here, we show that our PEO-PDLLA nanoparticles display a monomodal size distribution with an intensity-average hydrodynamic diameter of around 100 nm and zeta potential ~–14 mV. Thus, this nanoparticle formulation has characteristics that are favorable for packaging and delivering therapeutic payloads and bioimaging agents. Likewise, the formulation process of the PEO-PDLLA nanoparticles is ideal for the incorporation of highly water-insoluble molecules and compounds. This is a major advantage of the nanoparticles described here over other nanoparticle platforms. There are currently many promising therapeutic compounds for a diverse range of diseases that are not currently being pursued due to an unfavorable solubility profile³⁸.

In addition to the unique PEO-PDLLA nanoparticles, the utilization of TIPS pentacene is also a novel aspect of the present study. TIPS pentacene is a soluble organic semiconductor used for electronics and has not been previously used in biomedical applications. To this end, we investigated its efficacy in labeling nanoparticles for quantification and visualization of cellular uptake. We found that TIPS pentacene displays excitation and emission wavelengths that are optimal for nanoparticle assessments in cells using imaging flow cytometry. Because size and charge both play extremely important roles in uptake efficacy and previous reports have shown that the loading levels of imaging agents and drug in nanoparticles can affect their size^{2,26–28}, we also evaluated the size and net charge impacts of loading the PEO-PDLLA nanoparticles with TIPS pentacene. Our results showed that PEO-PDLLA nanoparticles maintained a hydrodynamic diameter of around 100 nm and ZP ~–14 mV that was insensitive to the TIPS pentacene target loading in the range of 1–2 wt%. Moreover, the fluorescent labeling was done by simply mixing the dye with the diblock copolymer prior to particle formation, thus avoiding the need to covalently couple the dye to the polymer. We conclude that TIPS pentacene-loaded PEO-PDLLA nanoparticles offer a novel avenue for biological imaging in the lung with little to no side effects. These nanoparticles are a significant improvement over several alternative formulations and imaging agents, including carbon nanomaterials and quantum dots, which have less favorable immunogenicity and cytotoxicity profiles.

The overall objective of our present study was to better define macrophage uptake of nanoparticles under clinically relevant, pathological conditions in the lungs. Similar to prior studies, we initially evaluated nanoparticle phagocytosis in M1 and M2 polarized macrophages. In prior studies, M2 macrophages have generally been considered to have increased nanoparticle phagocytosis compared to M1 macrophages^{3,11}. However, our data did not reveal any significant differences in PEO-PDLLA nanoparticle phagocytosis under

the conditions tested. While these findings seem contradictory to those previously reported, it is likely that the differences are associated with nanoparticle size, shape, and composition. For example, one of the features of the nanoparticles used in our present study is the presence of a brush of PEO chains that extend out from the particle surface into the solution. The dense packing of chains provides a hydrophilic PEO shell around the hydrophobic PDLLA core, resulting in particle stability and biocompatibility³⁹. By contrast, the lack of a hydrophilic brush can lead to nanoparticle aggregation, which may also underlie some of the prior differences reported for M1 and M2 macrophage up-take. M1 macrophages have been shown to be more efficient in phagocytosing larger nano- and micro-sized particles. Thus, aggregation may favor M1 up-take over M2. In addition to brush differences, the composition of nanoparticles can also dramatically affect up-take dynamics and has also been shown to significantly influence M1 versus M2 macrophage phagocytosis^{3,11–13}. For example, M1 macrophages appear to more readily phagocytose and internalize polystyrene nanoparticles^{12,13} while phagocytosis of PEO and PLGA-based nanoparticles appears to be more efficient in M2 macrophages, depending on size and shape³. Nanoparticle size can also dramatically influence macrophage up-take dynamics as clearly shown in the context of silica nanoparticles^{11,40}. In these prior studies, M1 macrophages showed greater up-take of silica nanoparticles that were approximately 158 nm in diameter; whereas, M2 macrophages showed increased phagocytosis of silica nanoparticles that were approximately 20–40 nm in diameter^{11,40}.

The ultimate goal of nanoparticle based drug delivery and detection systems is to improve therapeutic strategies associated with disease pathobiology. Thus, it is critical to understand the properties and behavior of nanoparticles not only under naïve conditions, but also in the context of the inflammatory environment where they are most likely to be utilized and targeted. In general, prior studies evaluating nanoparticle uptake and biodistribution have focused on these dynamics under naïve conditions or based findings predominately on *in vitro* studies^{1,32}. While these types of studies have been essential to characterize host-nanoparticle interactions and have laid the foundation for the field of nanomedicine, the *in situ* and clinical relevance is limited. Thus, here we sought to characterize macrophage uptake dynamics in the context of Th1 and Th2 mediated airway inflammation *in vivo*, which model clinical conditions that would most likely benefit from nanoparticle based therapeutics. Under these inflammatory conditions in the lung, macrophages recruited to the airways will polarize into M1 or M2 phenotypes, respectively, and exert a significant level of immune system modulation within their respective inflammatory microenvironments⁹. Thus, to evaluate differences in M1 and M2 nanoparticle uptake *in vivo*, macrophages were sorted from these mice and evaluated utilizing imaging flow cytometry. Consistent with our *ex vivo* findings, we did not detect a significant difference in the uptake of our TIPS pentacene loaded PEO-PDLLA nanoparticles between M1 and M2 macrophages. Interestingly, only a small percentage of macrophages from the Th1 and Th2 environments were positive for nanoparticles (approximately 6.5–10%). We believe that this is due, at least in part, to increased nanoparticle uptake by granulocytes in both models.

Overall, these data show that TIPS pentacene-loaded PEO-PDLLA nanoparticles have exciting potential for use in diverse biomedical applications. These nanoparticles were found to be immunologically inert and non-cytotoxic in both *in vivo* and *ex vivo* models. While

this means that these PEO-PDLLA nanoparticles alone would not be ideal as adjuvants, loading of other formulations with antigen has shown promise in acting as potent activators of dendritic cells and T cells¹⁸. Future studies using our nanoparticle formulation may include similar experiments. When considered along with other findings associated with M1 and M2 nanoparticle uptake dynamics, our data demonstrate the importance of considering not only nanoparticle characteristics, but also specific aspects of the host cell and model system in characterizing host-nanoparticle interactions. Together, these data further contribute to the design of novel nanoparticles targeting a wide range of pulmonary diseases and will hopefully contribute to future improvements in therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PEG	polyethylene glycol
Th1	T helper cell type 1
Th2	T helper cell type 2
AAMs	alternatively activated macrophage
PLGA	Poly(lactide-co-glycolide)
LiLa	lipid-latex hybrid
PtdSer	phosphatidylserine
9-CCN	cholesterol-9-carboxynonanoate
PEO-PDLLA	Poly(ethylene oxide-b-D,L-lactide)
THF	tetrahydrofuran
TIPS pentacene	6,13 bis(triisopropylsilylethynyl) pentacene
DLS	Dynamic light scattering

BALF	Bronchoalveolar Lavage Fluid
BMDMs	Bone marrow derived macrophages
LDH	Lactate dehydrogenase
OVA	ovalbumin
LPS	lipopolysaccharide
i.t.	Intratracheal
PBS	phosphate buffered saline
H&E	hematoxylin and eosin
ELISA	enzyme linked immunosorbent assay
rtPCR	reverse transcriptase polymerase chain reaction
alum	aluminum hydroxide
ANOVA	Analysis of Variance
SEM	standard error of the mean
FOV	fields of view

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Figure 1. Characterization of PEO-PDLLA Nanoparticles and TIPS Pentacene

A) Intensity-average hydrodynamic diameter, polydispersity index (PDI) and zeta potential of PEO-PDLLA nanoparticles at different TIPS pentacene loadings. **B)** Normalized UV-visible spectroscopy absorbance and emission spectra of TIPS pentacene. **C)** Intensity-average hydrodynamic diameter, polydispersity index (PDI) and zeta potential of PEO-PDLLA nanoparticles at different TIPS pentacene loadings. *Standard deviations based on propagation of error analysis. [†]Standard deviations calculated on the basis of 5 measurements per sample.

Figure 2. M1 and M2 Macrophages Phagocytose PEO-PDLLA Nanoparticles Similarly Ex Vivo

A) Relative expression of *Tnf*, *Ifng*, *Ym1*, *Fizz*, and *Arg1* in bone marrow-derived macrophages after overnight polarization using IFN γ (M1) or IL-4 (M2). **B)** Percentage of M1 and M2 macrophages positive for PEO-PDLLA nanoparticles as measured by imaging flow cytometry. Macrophages were treated with nanoparticles for 2, 4, 8, 24 and 48 hours. **C–D)** Representative flow cytometry images of nanoparticle uptake in M1 and M2 macrophages. At least 10,000 cells were quantified for each sample. *, \$, and # indicate $p < 0.05$.

Figure 3. PEO-PDLLA Nanoparticles are Non-Immunogenic and Non-Cytotoxic in M1 and M2 Macrophages

Bone marrow-derived macrophages were polarized with IFN γ (M1) or IL-4 (M2) and treated with TIPS Pentacene loaded PEO-PDLLA nanoparticles for 2, 4, 8, 24 and 48 hours. LPS was used as a positive control. IL-6 (A–C) and TNF (D–F) levels were quantified via ELISA. G–H)

Figure 4. Induction of a Th1 Microenvironment in the Lung

A) Mice were treated with PBS, LPS, nanoparticles, or both LPS and nanoparticles via i.t. administration. LPS was given 24 hours before treatment of nanoparticles to elicit a Th1 environment in the lungs. **B)** Cytospins of the BALF were evaluated via differential cell counting. **C)** Representative histopathology images (20X) of lungs after H&E staining with scale bars of 0.01 mm. **D)** Histopathology was assessed and specific parameters associated with disease pathogenesis were scored to generate a composite histological activity index (HAI Score). **E)** Local IL-6 concentrations were determined via ELISA using BALF. n=9 for

LPS treated mice and n=7 for PBS treated mice. *, \$, # and $\neq$ indicate $p < 0.05$. N.D. = “not detected”.

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Figure 5. PEO-PDLLA Nanoparticles Are Phagocytosed By Immune Cells in Th1 Microenvironments in the Lung

Twenty four hours after nanoparticle treatment of LPS challenged mice, animals were euthanized and nanoparticle up-take was evaluated using imaging flow cytometry assessments of cells isolated from BALF. **A)** The number of cells positive for TIPS pentacene-loaded PEO-PDLLA nanoparticles were calculated for mice exposed to either PBS or LPS and treated with either PBS (vehicle) or nanoparticles (NP). **B)** Representative flow cytometry images of nanoparticles inside cells isolated from BALF. n=9 for LPS

treated mice and n=7 for PBS treated mice At least 10,000 cells were quantified and imaged for each animal. *, \$, and # indicate $p < 0.05$.

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Figure 6. Induction of a Th2 Microenvironment in the Lung

A) Mice were given intraperitoneal (i.p.) injections of OVA on days -21 and -7. Allergic inflammation was then induced via intranasal (i.n.) administration of OVA on days 1-5. Twenty-four hours following i.n. administration, mice were either given PBS or nanoparticles via i.t. administration. Tissues were harvested on day 7. **B)** Cytospins of the BALF were evaluated via differential cell counting. **C)** Representative histopathology images (20X) of lungs after H&E staining with scale bars of 0.01 mm. **D)** Histopathology was assessed and specific parameters associated with disease pathogenesis were scored to

generate a composite histological activity index (HAI Score). **E)** BALF was collected and analyzed for IL-6 concentration via ELISA. n=7 mice per experimental group. *, \$, and # indicate $p < 0.05$.

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Figure 7. PEO-PDLLA Nanoparticles Are Phagocytosed By Immune Cells in Th1 Microenvironments in the Lung

Twenty-four hours after nanoparticle treatment of OVA challenged mice, animals were euthanized and nanoparticle up-take was evaluated using flow cytometry assessments of cells isolated from BALF. **A)** The number of cells positive for TIPS pentacene-loaded PEO-PDLLA nanoparticles was calculated for mice exposed to either PBS or OVA and treated with either PBS (vehicle) or nanoparticles (NP). **B)** Representative flow cytometry images of

nanoparticles inside cells isolated from BALF. n=7 mice per experimental group. At least 10,000 cells were quantified and imaged for each animal.

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Figure 8. No Significant Difference in Macrophage Phagocytosis of TIPS Pentacene Loaded PEO-PDLLA Nanoparticles in Th1 and Th2 Microenvironments

A) Nanoparticle up-take was evaluated using flow cytometry assessments of selected macrophage populations isolated from BALF following either LPS or OVA exposure. The percent of macrophages containing nanoparticles was calculated. While both groups treated with nanoparticles (NP) were significantly increased versus the PBS (vehicle) treated animals, no significant differences were detected between macrophages harvested from LPS or OVA exposed mice. $n=9$ for LPS treated mice and $n=7$ for OVA and PBS treated mice. At least 10,000 cells were quantified and imaged for each animal. * and \$ indicate $p<0.05$.