Protein-avoidant ionic liquid (PAIL)–coated nanoparticles to increase bloodstream circulation and drive biodistribution

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The rapid clearance of intravenously administered nanoparticles (NPs) from the bloodstream is a major unsolved problem in nanomedicine. Here, we describe the first use of biocompatible protein-avoidant ionic liquids (PAILs) as NP surface modifiers to reduce opsonization. An ionic liquid choline hexenoate, selected for its aversion to serum proteins, was used to stably coat the surface of poly(lactic-co-glycolic acid) (PLGA) NPs. Compared with bare PLGA and poly(ethylene glycol)–coated PLGA particles, the PAIL-PLGA NPs showed resistance to protein adsorption in vitro and greater retention in blood of mice at 24 hours. Choline hexenoate redirected biodistribution of NPs, with preferential accumulation in the lungs with 50% of the administered dose accumulating in the lungs and <5% in the liver. Lung accumulation was attributed to spontaneous attachment of the PAIL-coated NPs on red blood cells in vivo. Overall, ionic liquids are a promising class of materials for NP modification for biomedical applications.

INTRODUCTION

Nanomedicine offers an alluring promise for drug delivery—the capacity to administer therapeutics to specific parts of the body while minimizing off-target effects seen with systemic administration of drugs, particularly chemotherapeutics (1). However, despite decades of excellent work in the field, only a handful of nanoparticles (NPs) have made it through clinical trials (2). One large barrier to translation of nanomedicines is the removal of the NPs from the bloodstream (3). Once injected intravenously, the NPs encounter a diversity of serum proteins that adhere to the surface of the particle to form a protein corona (4), alerting the immune system to a foreign invader, which then swiftly removes the vast majority of the injected NPs to the filtering organs, primarily the liver (5), for clearance from the body.

The current gold standard for reducing NP clearance is the use of poly(ethylene glycol) (PEG) coatings, which reduces clearance by increasing the hydrophilicity of the surface of the particle, thwarting the attachment of the circulating proteins (6). However, the widespread usage of PEG in many consumer products, and the simplicity of its chemical structure, has led to an estimated 25% of the general population developing anti-PEG antibodies (7), mainly anti-PEG immunoglobulin M (8). A recent clinical review of the immunogenicity of PEG included a report of the use of PEGylated uricase for the treatment of critical gout. Of 169 human patients, 89% developed high anti-pegloticase antibody response (9), which resulted in rapid clearance and injection-site reactions after the first injection, overwhelming any beneficial effect of the administered therapeutic. Therefore, strategies to avoid NP clearance that require alternatives to PEG are needed in the toolbox of nanomedicine.

Ionic liquids (ILs), which consist of asymmetric, bulky cations and anions that have melting points below 100°C (10), have been used across a broad range of applications, including synthesis (11), catalysis (12), and battery applications (13). They have a number of appealing properties, one of which is inherent tunability, where a small change in the chemical structure of one of the components results in a measurable shift in observed bulk properties (14). When synthesized from biocompatible materials, ILs have shown great promise in biomedical applications (15), including stabilizing proteins (16), drug delivery through the skin (17–19), and oral drug delivery (20), where the components of ILs can be selected to optimize physiologically relevant properties, such as membrane interaction (21). ILs have been used to coat NPs in nonbiological settings, such as catalysis (22) and sensing platforms (23), and some preliminary studies have evidenced their suitability in improving the biocompatibility of existing carriers (24). However, the versatility and tunability of ILs have not yet been exploited in the context of coating NPs to reduce opsonization in intravenous drug delivery. We hypothesized that ILs can be designed to coat NPs to reduce protein adsorption and opsonization. This hypothesis was inspired by the success of zwitterionic surfactants in reducing surface protein adsorption (25, 26). Here, we developed and synthesized a library of biocompatible ILs and screened for those that do not solvate proteins well—so-called protein-avoidant ionic liquids (PAILs). Upon incubation with NPs, PAILs spontaneously coat the surface of NPs with PAILs and form a stable coating. PAIL coating delays protein adsorption on the NP surface and reduces clearance of intravenously injected NPs in vivo.

RESULTS

NP modification

Poly(lactic-co-glycolic acid) (PLGA) was chemically conjugated to 1,1’-dioctadecyl-3,3,3’,3’-tetramethyldiindocarbocyanine, 4-chlorobenzenesulfonate salt solid (DiD) dye, and PLGA NPs were synthesized by surfactant-free nanoprecipitation to generate “bare” control NPs (see Methods). PEG was chemically conjugated to PLGA NPs to generate PEGylated-PLGA NPs. A library of choline-based ILs was synthesized to coat NPs (table S1). Choline was used as a cation because of its biocompatibility and prior history of use in humans. Choline is a dietary supplement and closely related to a neurotransmitter acetyl choline (27). Various anions were used to synthesize ILs. A range of carboxylic acid–based anions were tested, with carbon chain lengths of four to eight and with varying unsaturation, ring structures, and...
The number of oxygen atoms. Ion ratios of 1:1 to 1:6 (cation:anion) were also tested. ILs were allowed to coat NPs through a 3-hour incubation with stirring, followed by filtration. The synthesized NPs were then characterized with nuclear magnetic resonance (NMR) spectroscopy, dynamic light scattering (DLS), and transmission electron microscopy (TEM; for the choline hexenoate 1:2 particles). The ability of ILs to coat NPs was confirmed by NMR (presence of IL components), DLS (changes in NP sizes), and TEM for the lead IL, choline hexenoate.

The capping ability of ILs varied greatly with their composition. ILs were considered to have successfully capped the PLGA NP if, after the modification and filtration process, monodispersed [polydispersity index (PDI), <0.3] peaks appeared by DLS with hydrodynamic diameters <300 nm. ILs with ring structures, presence of oxygen atoms beyond the carboxylic acid functional group, and unbranched carbon chains shorter than six were not able to stably cap the NPs. Ion ratio also affected NP capping. Among the ILs with eight carbon anions, 1:2 octanoic acid created much smaller particles than its 1:1 counterpart. In the case of geranic acid, all ion ratios produced stably capped particles except 1:6. Figure 1 shows the full characterization of particles coated with choline hexenoate 1:2.

Presence of choline hexenoate on NPs was confirmed by proton NMR (Fig. 1A). From integration of the peaks corresponding to the choline and hexenoate, each component appears in molar equivalency—i.e., 1:1, despite adding a 1:2 ratio to the synthesis procedure. Addition of choline hexenoate slightly increased the diameter of the NPs as measured by DLS (Fig. 1B). Bare PLGA NPs (black) had an average diameter of 64.60 ± 9.41 nm (PDI = 0.135). PEGylation increased the NP diameter to 123.1 ± 14.8 nm (PDI = 0.2) (blue). IL-NPs (red) exhibited the largest diameter of 170.0 ± 19.1 nm (PDI = 0.16) (Fig. 1C). Corresponding changes were also observed in the surface charge of the NPs. Bare NPs (black) had the average zeta potential of −11.0 ± 4.03 mV. PEGylated NPs (blue) had a zeta potential of −31.3 ± 5.86 mV. IL-NPs (red) exhibited the most negative surface charge of −40.5 ± 2.12 mV. TEM indicated substantial changes in the morphologies of NPs (Fig. 1D). Bare PLGA NPs exhibited uniform composition, and size of NPs generally matched those determined from DLS. PEGylated NPs exhibited a high-contrast core surrounded by a ring of lower contrast around the PLGA core (Fig. 1E). IL-NPs exhibited a peculiar structure where the high-contrast core was surrounded by a much larger core of lighter contrast than the polymeric core (Fig. 1E), evidencing the successful surface modification of the PLGA NPs. Figure S4 shows the population size distribution (BD) by TEM. The IL-NPs retained this size and negative surface charge with an acceptable PDI for >3 weeks (figs. S1 to S3), only falling apart at 3.5 weeks, at which point the ester linkages in the PLGA likely begin to undergo hydrolysis (28), as seen by the PDI of the bare particles approaching 1 at the 3.5-week mark. This is consistent with earlier reports of 50:50 PLGA particles undergoing in vitro degradation after 21 to 26 days (29).

**In vitro assessment of NPs**

The serum stability and compatibility of IL-NPs were tested by incubating them with whole-mouse serum. IL-NPs maintained their stability in serum for various periods of time depending on the IL composition (table S2). IL-NPs resisted size increase for a composition-dependent time period, followed by a monotonic size increase. The duration of no size growth was considered as an indicator of the ability of IL-NPs to resist protein corona formation.

The identity of the anion played a significant role in protecting the NP from protein adsorption. Anions with carbon chains six to eight carbons in length emerged as the best at resisting protein adsorption. The role of the double bond emerged as a critical factor—the

![Fig. 1. An ionic liquid was used to successfully coat PLGA NPs. (A) $^1$H NMR spectrum of PLGA NPs surface coated with choline hexenoate (1:2). (B) DLS reported hydrodynamic diameter of NPs (log$_{10}$ transformation on the x axis) when bare (black), coated with PEG (blue), and coated with IL (red) with mean diameters above each curve. (C) DLS reported zeta potential of NPs when bare (black), coated with PEG (blue), and coated with IL (red) with mean diameters above each curve. (D) TEM of bare PLGA particles. (E) TEM of PEG-capped particles. (F) TEM of IL-coated particles. Scale bars, 200 nm.](image)
addition of a double bond to hexanoate to create hexenoate improved the resistance to serum by a factor of 22. In the case of the serum test, the role of the ion ratio is anion dependent. For the hexenoate, the 1:2 ratio outperforms the 1:1 IL by sixfold, but for geranic acid, the 1:1 ratio outperforms the others by twofold.

The most successful candidate of the library of ILs tested was choline hexenoate (1:2). Therefore, it underwent further testing and is hereafter referred to as PAIL.

Stability of NPs against aggregation was tested in undiluted mouse serum in a timed DLS test. The diameter of bare PLGA particles, shown as black diamonds, increased within minutes to 200% of their original size (Fig. 2A). This is consistent with numerous reports on the formation of protein coronae on polymeric particles (6, 7, 30). The PEGylated particles (blue circles) fared better than the bare NPs, only growing 60% larger than their original measured size in buffer over 30 min. The PAIL-coated particles (red squares) exhibited no appreciable size change even after 60 min in neat serum, only changing size after 90 min. The surface charge of the particles was measured at the conclusion of the serum test (Fig. 2B). The surface of the bare particles is very close to neutral, while both the PEG and PAIL show zeta potentials of −15 mV. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was performed to qualitatively investigate the degree of protein adsorption after 2 and 20 min in neat serum, evidencing that the PAIL-coated particles show a significantly smaller degree of protein adsorption. In vitro hemolysis of the particles was assessed with isolated mouse red blood cells (RBCs) (Fig. 2C). All particles showed minimal (<10%) hemolysis [no significant difference by analysis of variance (ANOVA) analysis, \( P = 0.96 \)], indicating their suitability for further intravenous testing.

**In vivo assessment of NPs**

PAIL-NPs were administered intravenously via tail vein into healthy BALB/c mice (Fig. 3A). Bare NPs exhibited rapid clearance with only 20% NPs remaining in circulation after 20 min (black diamonds). PEGylated NPs exhibited improved circulation over bare NPs with 14.2 ± 2.7% NPs remaining in circulation after 24 hours. PAIL-NPs exhibited the highest circulation with 23.6 ± 0.86% NPs remaining in circulation after 24 hours (\( P = 0.02 \) between PEG and PAIL-NPs). At the conclusion of the 24 hours, the major organs were excised, examined by IVIS (in vivo imaging system) imaging (Fig. 3C), and quantified by fluorescence spectroscopy (Fig. 3B).

In the case of the bare particles (Fig. 3B, black), 50% of the administered dose accumulated in the liver. Most of the PEGylated particles (blue) were also found in the liver 24 hours after injection. The PAIL-NPs, however, were predominately located in the lungs, with 50% of the administered dose accumulating in the lungs. High lung/liver ratio was observed for PAIL-NPs (~12), which was >200-fold greater than that for bare NPs or PEGylated NPs. IVIS images confirmed high lung accumulation of PAIL-NPs (Fig. 3D). The IVIS images of the major organs of all six mice across the treatment groups can be found in figs. S5 to S7. Note that the fluorescence that appears in the brain across all treatment groups is likely to be autofluorescence and does not reflect any accumulation of NPs in the brain.

Systemic toxicity of IL-NPs was assessed by measuring interleukin-6 (IL-6) in the blood to investigate the possibility of inflammation...
induced by NPs 24 hours after injection. Both the bare and PEGylated particles show significant levels of IL-6 at a concentration of ca. 100 pg/ml, while the PAIL-NPs show >2-fold lower values at <40 pg/ml (Fig. 3C).

**PAIL-RBC interaction drives BD**

To investigate the reason for high lung accumulation of PAIL-NPs, RBCs from mice were evaluated by both SEM and fluorescence-activated cell sorting (FACS). Figure 4 (A and B) shows the RBCs after injecting bare (A) and PEG-PLGA particles (B). RBCs harvested from mice injected with PAIL-NPs indicated presence of NPs on the RBC surface (Fig. 4C). These NPs were not seen in mice injected with bare NPs (Fig. 4A). RBCs were also incubated with PAIL-NPs in vitro and exhibited attachment (Fig. 4D). The imaged RBCs and plasma were also analyzed using FACS. Figure 4 (E and F) shows the results for RBC and plasma after injection of bare PLGA particles, where no fluorescence is observed when gated for the DiD dye in the RBC fraction, but a large amount of fluorescence appears in the plasma. PAIL-NPs, however, exhibited high attachment to RBCs (Fig. 4G) and minimal presence in the plasma (Fig. 4H).

**DISCUSSION**

The clearance of NPs from the bloodstream after intravenous injection remains a pressing issue even as new, promising, nanomedicines are reported near daily. Agents other than PEG are needed to overcome the immune challenges generated by its overuse. PLGA NPs were successfully coated with choline hexenoate to generate PAIL-NPs (Fig. 1). The PAIL-NPs exhibit a larger hydrodynamic radius and a more negative surface charge compared with the bare and PEGylated particles, indicating successful modification. The PLGA core has a slight negative surface charge, meaning that the layer closest to the core consists of the cation. The final, negative surface charge of the particle indicates that the terminal layer consists of anions.

The arrangement of ILs at a charged surface is a well-known phenomenon in contexts such as electrochemistry, where the ionic components arrange spontaneously in an alternating fashion at a charged surface, such as an electrode, to form an electrical double layer. Atkin and Warr (31) used atomic force microscopy to analyze ILs at mica, silica, and graphite surfaces, finding that the cations and anions arranged in alternating layers and that each layer was approximately 1 nm thick. This modification process can be likened to a layer-by-layer approach (32), except in this case, the process happens in one pot and is controlled by electrostatics rather than sequential addition of materials with alternating charges.

Zwitterionic polymers have been previously shown to exhibit stealth capabilities (26, 33). Specifically, quaternary ammonium-carboxylic acid–terminated zwitterions have been used to successfully resist fouling in 50% fetal bovine serum (25). ILs offer a modular material that offers outstanding tunability of parameters. They, thus, offer an excellent addition to the available set of tools for controlling NP surface properties.

The NMR data show, through quantitative peak integration using tetramethylsilane, that despite adding a 1:2 ratio of cation:anion, the
IL modifies the NP at an equal molar ratio of 1:1. When a 1:1 ratio is added in the case of choline hexenoate, the resulting coating is less able to resist the serum proteins, suggesting that the excess of anion confers a benefit when self-assembling around the PLGA core in the case of this anion.

The results of the in vitro serum test indicate that the PAIL-NPs outperform the conventional PEGylated particles. The primary mechanism of PEG’s ability to resist serum proteins has been as attributed to its hydrophilicity. The data reported here suggest that the way in which PAILs protect the NP is more complex. Compared with the lead candidate anion (hexenoate), hexanoate has the same structure but lacks the double bond in the 2 position. This simple change in anion composition resulted in significantly reduced ability of the capped NPs to resist serum adsorption. The removal of this double bond has a marked effect on its ability to protect the NP from serum proteins, with the choline hexanoate particles increasing in size by 178.4% within 10 min. Comparatively, the lead candidate choline hexenoate 1:2 is able to resist any size change in pure serum up to 90 min. We hypothesize that the protein avoidance comes from poor solvation of the proteins, such that the proteins actively avoid interacting with the IL layering around the NP. Anions that contain oxygen atoms (other than the carboxylic acid functional group) performed significantly more poorly in capping ILs than those without additional oxygen atoms. This may be due to specific interactions occurring between the electronegative oxygen atoms and pockets of the proteins. Solvation of cytochrome c has previously been reported for choline dihydrogen phosphate by Forsyth et al. (34), where they hypothesize that its ability to act as both a hydrogen bond acceptor and donor enables its capacity to stabilize the protein, including its secondary structures.

At the conclusion of the serum test, the surface charge of the particles was measured. The bare particles showed neutralized surfaces, while both the PEGylated and PAIL-NPs retained negative zeta potentials of ca. –15 mV. The serum-exposed particles were then examined by SDS-PAGE after 2 and 20 min of exposure. Across all molecular weights, the PAIL-NPs show very faint or nonexistent bands, indicating that the PAIL-NPs have successfully lowered protein adsorption relative to the bare and PEG controls. This experiment was carried out by mixing the NPs with pure serum, which exposes the NPs to a very high concentration of protein, resulting in significant adhesion even in the case of the PEGylated particles.

The low levels of hemolysis of mouse RBCs in vitro (<10%) suggest that the PAIL-NPs do not induce cell lysis, which is an important factor in developing new agents delivered intravenously. More broadly, consideration of toxicity is critical when selecting ILs to screen, and as such, the components chosen to make the ILs were selected to ensure that they have high biocompatibility. The lead anion, trans-2-hexenoic acid, is on the Generally Regarded as Safe list and is used as a food additive to alter flavor and fragrance (35).

The in vivo testing of the NPs in healthy BALB/c mice showed that, once again, the PAIL-NPs outperformed both the PEGylated and bare controls, showing the greatest retention in the bloodstream of the mice at 24 hours. The use of PAIL-NPs instead of the control NPs resulted in a >2-fold reduction in inflammatory cytokine IL-6, suggesting the particles were far less immunostimulatory than the controls. In addition, the use of the PAIL coating substantially altered the BD of the particles, leading to a significant reduction in hepatic accumulation at 24 hours. Instead, 50% of the administered dose appeared in the lung tissue, with a lung:liver ratio of ca. 12 for the PAIL-NPs. Taking a closer look at RBCs removed from the mice 24 hours after injection using electron microscopy reveals the mechanism of this altered BD. RBCs examined from PAIL-NP–treated mice show both adhered NPs and shear marks on the surface of the RBC. No adherence is seen in RBCs removed from mice in the other treatment groups. When PAIL-NPs are exposed to RBCs in vitro, 50% of the NPs added appear attached to the surface of the RBCs.
Our hypothesis for this phenomenon is that given the poor solvation of the serum proteins, the RBCs are the most abundant surface that the NP comes into contact with after injection. The precise mechanism for the interaction between the RBCs and the PAIL-NPs is yet to be uncovered, but a working hypothesis is that the terminal anionic layer of the PAIL-NPs interacts electrostatically with the abundant cationic lipids (namely, phosphatidylcholine and sphingomyelin, which are very structurally similar to the cation of the IL) in the outermost layer of RBCs. This kind of electrostatic interaction has been reported before in the case of negatively charged silica NPs, where the interaction of the NPs with RBC surfaces resulted in hemolysis (36). However, the alternating charge composition of the IL-NPs do not result in a high degree of hemolysis, perhaps because the cationic layer of IL is able to shield the terminal anionic layer, which prevents the IL-coated NP from entering the membrane to the point of rupture.

The data presented in Fig. 4 strongly suggest that PAIL-NPs attach to the surfaces of RBCs in the bloodstream after injection and are systematically circulated until they encounter the dense capillary beds, which shear the particles off into the endothelium of the surrounding blood vessels (37). When injected into the tail vein, the lung tissue is the first dense capillary bed encountered by circulating RBCs. This "hitchhiking" phenomenon has been reported previously (37), with two substantial differences. First, PAIL-NPs spontaneously adhere to the RBCs in the bloodstream, while previous methods required an ex vivo treatment to observe attachment. Second, the PAIL-NPs remain in the lung tissue at 50% of the injected dose at 24 hours and are not cleared by the RES (reticuloendothelial system), while previous hitchhiked NPs only showed preferential accumulation after 6 hours of administration.

The results presented here show the potential of ILs in enhancing the capabilities of NPs for drug delivery, especially given their large compositional diversity (11) and the high degree of tunability. Many exciting questions and possibilities need future attention, including understanding the specific interactions between the ILs and the proteins in the serum and the mechanisms of their interactions with the RBC surface. With further research focused on mechanisms and safety, IL-coated NPs open new opportunities in drug delivery.

Conclusions
Here, we report the first use of biocompatible ionic solvents as NP surface modifiers for reducing opsonization. Choline hexenoate emerged as the leading PAIL, stably attaching to the surface of PLGA particles. The PAIL-NPs showed excellent resistance to protein adhesion and, when administered in vivo, provided greater retention in the bloodstream compared with PEGylated and bare NPs. PAIL also substantially altered the BD of the NPs, with 50% of the administered dose accumulating in the lungs. Electron microscopy of RBCs at the conclusion of the experiment showed that this altered BD was a result of the PAIL-NPs spontaneously adhering to the RBCs in the bloodstream. Overall, we demonstrate the potential of the use of ILs as materials in NP drug delivery.

MATERIALS AND METHODS

Materials
Choline bicarbonate, trans-2-hexenoic acid, acetonitrile [high-performance liquid chromatography (HPLC) grade, 99.8% purity], phosphate-buffered saline (PBS), D₂O, Resomer RG 504 H, poly(D,L-lactide-co-glycolide) 50:50 (PLGA) with molecular weight (MW) 38,000 to 54,000 kDa and that was carboxylic acid terminated, PEG (MW 8000), and tetramethylsilane were obtained from Sigma-Aldrich (St. Louis, MO). DiD was obtained from Thermo Fisher Scientific.

Methods

Synthesis of ILs
Various carboxylic acids dissolved in ultrapure water were reacted with choline bicarbonate in a specified molar ratio (cation:carboxylic acid) at 40°C for 12 hours. A rotary evaporator was then used to dry the resulting ILs at 20 mbar at 60°C for 2 hours. The residual water was removed under a pressure of (~760 mmHg) at 60°C for 48 hours. 1H NMR characterization was performed and was found to be consistent with the previously published spectra (19).

NP core synthesis
PLGA was dissolved into HPLC-grade acetonitrile (ACN) at 1 mg/ml and vortexed for 5 min to ensure homogeneity. The far-red fluorescent dye DiD was then added into the PLGA/ACN organic phase solution at a concentration of ~1% (maximum capacity, 3%) by mass of PLGA. The final organic-phase solution was observed to turn a bright light-to-medium turquoise blue color after vortexing for another 5 min to suspend the DiD in the PLGA solution.

To synthesize uncapped PLGA NPs containing fluorescent DiD dye, 2 ml of the previously described organic-phase [1 mg PLGA/1 ml ACN] with 1% by mass PLGA fluorescent dye] formulation was added slowly dropwise to the aqueous phase (3 ml of Milli-Q water or 3 ml of D₂O for 1H NMR analysis) and allowed to stir in open air for 3 hours in the dark (38). After 3 hours of mixing in open air to achieve solvent evaporation, the uncapped NP solution was then removed from the magnetic stirring apparatus and stored in the dark at 4°C in a 1.5-ml centrifuge tube.

Synthesis of PEG-capped PLGA NPs (PEG-PLGA-DiD NP)
To synthesize PEG-capped PLGA NPs containing DiD dye, PEG was pre-added to the aqueous phase (3 ml of Milli-Q or D₂O water) at a [2:5:1] PEG:PLGA mass/mass ratio between the PEG in the aqueous phase and PLGA in the added organic phase. This aqueous solution was left to mix for 30 min in open air in the dark via a magnetic stir bar in the round-bottom flask. Two milliliters of the previously described organic-phase PLGA formulation was then added slowly dropwise to the aqueous phase and allowed to stir in open air for 3 hours.

Synthesis of IL-capped PLGA NPs (PAIL-PLGA DiD NP)
After the uncapped NPs were synthesized, they were stored in the dark in 1.5-ml centrifuge tubes at 4°C for about 1 hour to stabilize before neat-synthesized IL was added all at once via pipette directly to the center vortex of a solution of uncapped NPs at a concentration of 10 mg neat IL/mg PLGA under magnetic stirring conditions in open air and allowed to mix for another 3 hours to allow for the PLGA surface self-assembly capping process.

NP filtration and storage
Both PEGylated and PAIL-modified NP solutions were then centrifuged filtered in Amicon Ultra-4 centrifugal filter units, 10K MWCO (Millipore, 4 ml) at 3000 rpm for 50 min to eliminate unbound IL or PEG (8 kDa) in the NP solution. The resultant filtered NPs were then washed in MilliQ, 1× PBS (pH 7.4), or D₂O water and filtered at the same parameters. The final filtered NPs were then brought up to 1 ml in D₂O or MQ-H₂O for in vitro characterization. NPs were brought up to 1 ml with 1× PBS (pH 7.4) for in vitro physiological/biological work or 0.9% isotonic physiological saline for immediate use in vivo work and stored in the dark at 4°C in 1.5-ml centrifuge tubes.
Characterization of surface-modified NPs

NMR spectroscopy

Spectra were recorded on an A600a Agilent DD2 600 MHz NMR spectrometer. Each sample contained 1000 μl (2 mg) of NPs in D2O. Neat tetramethylsilane (TMS) was used additionally for quantitative NMR as an internal reference standard at a known amount of 4.5 mg (density = 0.648 g/ml at 25°C, MW 88.22 g/mol).

DLS was performed on a Zetasizer ZEN3600 (Malvern, UK) in a disposable polystyrene cuvette (hydrodynamic radii) or DTS1070 (zeta potential) cell at 25°C. Each sample (100 μl) was diluted up to 1000 μl (1:10) in H2O.

Quantification of fluorescence was achieved with a Spectramax i3 plate reader (Molecular Devices, San Jose, USA) using Corning Costar 96-well assay black and clear bottom plates (#3603) at excitation/ emission wavelengths of 644 and 670 nm. A calibration curve was obtained by serial dilution with acetonitrile over a concentration range of 0.01 × 10 −9 to 9.54 × 10 −9 mg/ml. Each well contained 200 μl. The experimental samples were measured in quadruplicate. TEM was performed via a Hitachi 7800 TEM at 80.0 kV using copper TEM grids (Electron Microscopy Sciences, carbon film 300 mesh copper). The samples were prepared by drop-casting a diluted NP solution (2 μl/1 ml of MQ-H2O), negative staining with 2% uranyl acetate, and washing with MQ-H2O, and stored in a dry and dark TEM grid box for 24 hours.

Assessing surface-modified NPs in vitro

NP size kinetics in neat mouse serum (by DLS)

Disposable polystyrene cuvettes were used in a Malvern Zetasizer at 25°C to monitor the size changes of the NPs in mouse serum. Control spectra were recorded both for the NPs at 1:10 dilution in MQ-H2O and neat mouse serum. One hundred microliters of surface-unmodified, PEG-coated, and IL-coated PLGA NP samples was separately diluted up to 1000 μl (1:10) in neat whole commercial mouse serum (Invitrogen, #10410). Each sample’s size in serum was measured from 2 to 100 min and actively examined for any size shifts every ~4 to 6 min from the original control (MQ-H2O) peak population. This continued until the NP population peak in serum was observed to begin shifting toward 200+ nm from the original MQ-H2O NP reference peak, indicating the starting point of protein adsorption and protein corona formation.

NP zeta potential kinetics in neat mouse serum

NPs were diluted (100:1000 μl) in whole normal mouse serum and incubated at 25°C up until the terminal size point, with occasional gentle pipette aspiration and ejection to simulate in vivo flow conditions. To minimize damage to the gold electrode plates in the zeta potential cell by whole neat serum, centrifugation was performed in reconstituted 2 ml of MQ-H2O each time, as a modified protocol by Partikel et al. (30), at 1500 rpm for 15 min each (30 min total per sample) to gently flush through unbound serum proteins and preserve PLGA NP integrity. Each sample’s final filtrate was then brought up to 1 ml in MQ-H2O, and zeta potential values were respectively measured in cleaned and prepared zeta potential DTS1070 cells.

SDS-PAGE following incubation in neat mouse serum

NPs were mixed into whole normal mouse serum (200 μl NPs:800 μl neat serum) and incubated at 25°C for two time points—2 and 20 min. After treating with neat serum, each sample was centrifuged thrice for 15 min at 3000 rpm. After each centrifugation, 800 μl of the upper nonadsorbed serum phase was carefully removed and replaced with an equivalent volume of 1× PBS (pH 7.4), thoroughly mixed by pipetting, and allowed to rest for 5 min. A final, fourth, centrifugation was performed for 10 min for each sample, after which the final NP sample was carefully isolated and transferred to a fresh tube. Laemmli buffer was added to each sample at a 1:1 ratio to each sample and subjected to a 100°C degradation for 5 min. SDS-PAGE (12%, tris-Gly-SDS running buffer, 20-μl loading, Bio-Rad) was then performed for 30 min at 200 V. The gel was washed three times for 5 min each in 200 ml of MQ-H2O. Water was removed, and the gel was covered with ready-to-use Coomassie stain and shaken slowly for an hour. The gel was briefly washed with MQ-H2O again and then destained with 50% HPLC methanol solution and 10% glacial acetic acid solution until the background was removed and the ladder/bands were clearly visible. Last, the gel was washed with MQ-H2O again to remove the excess destaining solution and then imaged using an iPhone camera. Kinetic hemolysis assays were performed using a modified protocol adapted from Evans et al. (39), in which 250 μl of whole blood was exsanguinated from a wild-type adult BALB/c mouse 3 months of age immediately postmortem via CO2 induction and delivered into a K2-EDTA–coated vacuum tube to prevent coagulation. Whole blood was then centrifuged at 1000g for 10 min, and then plasma was discarded to isolate the RBC pellet. The RBC pellet was then restored to 250 μl with 1× PBS (pH 7.4) and centrifuged at 500g for 5 min. This was repeated twice more. Four hundred ninety microliters of 1× PBS (pH 7.4) was then pipetted into each of 4 ml × 1.5 ml conical tubes. Washed isolated erythrocytes (10 μl) were then added into each tube to produce a 1:50 dilution. These tubes constituted the stocks of diluted RBCs.

In a 96-well clear plate, each well received 20 μl of control or NP solution + 180 μl (1:10) of diluted RBC stock. The positive internal control was denoted as 20 μl of 20% Triton X-100 into 180 μl of diluted erythrocytes. The negative internal control was denoted as 1× PBS (pH 7.4) at the same dilution. The 96-well plate with samples was incubated at 37°C for 1 hour and then centrifuged at 500g for 10 min. After centrifugation, 100 μl of supernatant was collected and transferred from each treatment well into a new clear, flat-bottomed 96-well plate, which was then measured for peak absorbance at an experimentally determined 405 nm on a Spectramax i3 plate reader as a kinetic measurement over 2 hours at 25°C. For analysis of data, the absorbance readings from 1× PBS (pH 7.4) negative internal control (0%) were used to subtract background measurement for all samples. From there, all quadruplicate measurements were normalized as a percentage of hemolysis to the Triton X-100 positive internal control (100%).

Assessing surface-modified NPs in vivo

Animals

Female BALB/c mice (12 weeks of age) were purchased from Charles River Laboratories (MA, USA). All experiments were performed according to the approved protocols by the Institutional Animal Care and Use Committee of the Faculty of Arts and Sciences, Harvard University, Cambridge.

Biodistribution and pharmacokinetics. Healthy 3-month-old adult female BALB/c mice were used to evaluate pharmacokinetics (PK) and BD of the NPs. The study used the following four groups for BD and PK studies in healthy BALB/c mice: (i) ~70-nm spherical PLGA DiD particles (negative control), (ii) ~120-nm spherical PEG-PLGA DiD particles (positive control), (iii) ~180-nm spherical CAHA (Choline 2-hexenoate) 1:2 PLGA DiD particles, and (iv) 0.9% saline internal control injection. One hundred microliters of PLGA,
PEG-PLGA, and CAHA 1:2-PLGA NPs in 0.9% physiological saline (final concentration, ~1x10^11 NPs) was administered intravenously by tail vein injection in parallel for BD and PK purposes (n = 6). To perform pharmakokinetic studies, ~40 μl of blood per mouse was taken from the submandibular vein without injection (0 min) and immediately after (within 2 min) injection, as well as at 1, 6, and 24 hours. Extracted aliquots of blood during the study were analyzed on a fluorescence plate reader to quantify the number of NPs in circulation as a percentage of the administered dose. To do this, blood extracted was directly delivered into K2-EDTA–coated tubes and immediately stored in the dark at 4°C to efficiently preserve and quantify NP fluorescence.

After acquiring blood at the 24-hour time point, fluorescence for all samples' time points was measured as described above. To quantify the relative percentage of NPs remaining in circulation after injection at each blood sample time point, the background fluorescence [relative fluorescence unit (RFU)] of untreated blood (time 0) was first subtracted from all blood samples at 2 min, 1 hour, 6 hours, and 24 hours. After background subtraction, the raw fluorescence (RFU) at 1, 6, and 24 hours were then compared to that of 2 min (100% fluorescence of administered dose) to obtain the percentage of NPs remaining in circulation after injection over time.

At the 24-hour terminal blood sampling time point, each mouse was exsanguinated under isoflurane anesthesia to collect final aliquots of blood for postmortem characterization [FACS, enzyme-linked immunosorbent assay (ELISA), and SEM] and then immediately euthanized by CO2 to collect main blood-filtering organs (heart, kidneys, lung, liver, spleen, and brain) for the BD study. Following modified and combined protocols originally from Oliveira et al. (40) and McGowan and Bidwell (41), a BD study was performed to measure the accumulation of the NPs in the organs. Immediately after extraction after euthanasia, organs were preserved within 50-μl Falcon tubes in 4% methanol-free paraformaldehyde for 2 hours and then transferred to and washed several times in 1× PBS (pH 7.4). Immediately after washing in PBS, samples were then transferred to a petri dish, and IVIS small animal imaging was performed at 644 and 670 nm to visualize epifluorescence from the organs in units of radiant efficiency [(p/s/cm²/sr)/(μW/cm²)]. IVIS imaging was performed not only to visualize the presence of fluorescent NPs but also to analyze the particle concentration in the organs.

After visualization by IVIS, organs for each treatment sample were submerged in Falcon tubes in just enough radioimmuno-precipitation assay (RIPA) lysis buffer (RIPA Lysis and Extraction Buffer, G-Biosciences) to coat the surface of the tissue and homogenized at 30,000 rpm by the IKA handheld homogenizer (IKA Inc., T10 Basic S1, Ultra Turrax, 8000 to 30,000 rpm) until a homogeneous liquid was formed. After all treatment samples were homogenized, fluorescence (RFU) was quantified as described above. All data were analyzed using one-way ANOVA followed by post hoc Tukey.

FACS of RBCs. Isolated RBCs from the 24-hour samples were analyzed by FACS to qualitatively evaluate whether the NPs were adhering to blood cells or being targeted by macrophages in plasma for clearance, if still in circulation at 24 hours. An in vitro assay was constructed first to examine feasibility of this theory before examining in vivo.

For in vitro studies, a 1:10 ratio of NPs/RBCs corresponding to in vivo injections was prepared by mixing each NP treatment with whole mouse blood. First, whole blood was exsanguinated from a wild-type control BALB/c mouse under inhalatory anesthesia for 10 min, combined with the NP treatments, and then centrifuged at 1000g for 10 min to obtain isolated RBCs. These RBCs were then washed using 1x PBS (pH 7.4) three times with centrifugation at 200g for 10 min between each wash. The final samples were each resuspended in 3 ml of 1x PBS (pH 7.4).

For in vivo samples, a representative mouse from the PLGA-DiD, saline, and CAHA 1:2-PLGA-DiD NP treatment groups was selected for examination after 24 hours (blood withdrawal and euthanasia). RBCs were isolated from whole blood withdrawn from each mouse and washed three times in 1x PBS (pH 7.4) as previously described. In addition, supernatant was collected and examined as well for macrophage NP uptake activity. All in vitro and in vivo samples underwent FACS analysis on an LSR II Fortessa flow cytometry machine to examine the detection and location of any NPs (which emitted far-red fluorescence from encapsulated DiD dye) at 670 nm.

**SEM of RBC-NP interactions.** After FACS confirmed the presence of NPs adhering onto RBCs both in vitro and in vivo, SEM was performed on these same RBC samples on a Supra55 SEM machine according to the previously established protocol of Brenner et al. (37).

**Measured proinflammatory immune response (ELISA).** Sera derived from whole blood taken from the final 24-hour in vivo time point after intravenous injection were analyzed using protocol accompanying an ELISA kit to detect IL-6. To conduct the ELISA, 100 μl of serum from each treatment group was taken to establish the IL-6 response (Invitrogen Mouse IL-6 Uncoated ELISA Kit with protocol).

Absorbance was measured at 450 nm on a 96-well clear ELISA plate using a Spectramax plate reader (Spectramax i3). After acquisition, 4PL statistical fitting was applied to the ELISA mouse IL-6 standard curve, and two-tailed paired t test of the means was applied to assess significance between two treatment groups at a time.

**Statistical analysis.** Statistical analyses were conducted using Microsoft Excel 2016 for PK (n = 6) and BD (n = 6) data, which were both analyzed using one-way ANOVA followed by Tukey post hoc test and two-tailed paired t test of the means when interested in cross-verifying significance between two treatment groups. Background buffer autofluorescence was subtracted from all fluorescence-related measurements to account for true sample values. Fluorescence (RFU) was quantified by a SpectraMax i3 plate reader on 200 μl of homogenates at the same settings as prior to measure the percentage of NPs relative to the tail vein injection dose. 4PL statistical fitting via RStudio OpenSource was applied to the ELISA mouse IL-6 standard curve, and two-tailed paired t test of the means was applied to assess significance between two treatment groups at a time. All data are represented as means ± standard error of the mean. DLS is represented as a measure of intensity (%) for size or count for zeta potential. As an internal control, all DLS readings were measured with at least two internal readings/sample/trial.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/48/eabd7563/DC1

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